# Alanyl-Phosphatidylglycerol Synthase from *Pseudomonas aeruginosa*: Physiological relevance and mechanism of tRNA-dependent catalysis

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von Stefanie Hebecker, geb. Klein
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## Tagungsbeiträge

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*Jörg &*

*meiner Familie*

*Die schönste Freude erlebt man immer da, wo man sie am wenigsten erwartet hat.*

Antoine de Saint-Exupéry

# ABBREVIATIONS

| | |
|---|---|
| 2D-TLC | two-dimensional thin layer chromatography |
| $A_\lambda$ | absorption at wavelength λ nm |
| aa-PG | aminoacyl-phosphatidylglycerol |
| aa-PGS | aminoacyl-phosphatidylglycerol synthase |
| ACP | acyl carrier protein |
| AlaRS | alanyl-tRNA synthetase |
| $Ap^r$ | ampicillin resistance |
| A-PG | alanyl-phosphatidylglycerol |
| A-PGS | alanyl-phosphatidylglycerol synthase |
| APS | ammonium peroxodisulphate |
| ATP | adenosine triphosphate |
| AU | absorption units |
| BSA | bovine serum albumin |
| $Cam^r$ | chloramphenicol resistance |
| CAMP | cationic antimicrobial peptide |
| $Cb^r$ | carbenicillin resistance |
| CDP | cytidine diphosphate |
| CMP | cytidine monophosphate |
| CPI | chloroform phenol isoamyl alcohol |
| CTP | cytidine triphosphate |
| $C_v$ | column volume |
| DIPFP | diisopropyl fluorophosphate |
| DPG | diphosphatidylglycerol |
| ds | double stranded |
| (d)dNTP | (di)deoxynucleotide triphosphate |
| DTT | 1,4-dithio-D,L-threitol |
| ESI-MS | electrospray ionization mass spectrometry |
| *et al.* | *et alteri* (and others) |
| FA | fatty acid |
| FRT | flippase recognition target |
| *g* | earth gravity |
| GC/MS | gas chromatography/mass spectrometry |
| $Gm^r$ | gentamycin resistance |
| GST | glutathione *S*-transferase |
| $dH_2O$ | deionized water |
| HisRS | histidyl-tRNA synthetase |
| ICP-MS | inductively coupled plasma - mass spectrometry |
| IPTG | isopropyl-β-D-galactopyranoside |
| $Kan^r$ | kanamycin resistance |
| LB | Luria Bertani (medium) |
| LC-MS | liquid chromatography - mass spectrometry |
| λ | wavelength |
| L-PG | lysyl-phosphatidylglycerol |
| L-PGS | lysyl-phosphatidylglycerol synthase |
| MCS | multiple cloning site |

| | |
|---|---|
| MprF | multiple peptide resistance factor |
| $M_r$ | relative molecular mass |
| MS/MS | tandem mass spectrometry |
| MU | Miller unit |
| NMR | nuclear magnetic resonance |
| $OD_\lambda$ | optical density at wavelength λ nm |
| ORF | open reading frame |
| PBS | phosphate buffered saline |
| PE | phosphatidylethanolamine |
| PG | phosphatidylglycerol |
| $P_i$ | inorganic phosphate |
| $PP_i$ | inorganic pyrophosphate |
| p.p.m. | parts per million |
| pH | negative decadic logarithm of the $H^+$ concentration in a solution |
| PMSF | phenylmethylsulfonyl fluoride |
| p.s.i. | pounds per square inch |
| PVDF | polyvinylidendifluorid |
| rNTP | ribonucleotide triphosphate |
| rpm | rotations per minute |
| RT | room temperature |
| SAH | S-adenosylhomocysteine |
| SAM | S-adenosyl-L-methionine |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TAE | Tris-acetate-EDTA |
| TE | Tris-EDTA |
| TEMED | N,N,N',N',-tetramethyl ethylen diamine |
| TES | N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid |
| $Tc^r$ | tetracycline resistance |
| Triton X-100 | t-octylphenoxypolyethoxyethanol |
| Tween 20 | polyoxyethylensorbitanmonolaurat |
| U | unit |
| UV/VIS | ultra violet and visible spectrum of light |
| v/v | volume per volume |
| w/v | weight per volume |

# GLOSSAR

| | |
|---|---|
| microhelix | diminished tRNA molecule which consists of only the acceptor stem and a loop region |

# 1 INTRODUCTION

## 1.1 Membranes and Bacterial Lipid Homeostasis

Biological membranes separate the cell from the environment and build compartments inside the cell to divide and protect important cellular processes. Membranes are part of the bacterial cell envelope which can be differentiated between Gram-negative and Gram-positive bacteria. The cell envelope of Gram-positive bacteria contains a cytoplasmic membrane, which is covered by a thick peptidoglycan layer. In contrast, Gram-negative bacteria possess a thin peptidoglycan layer and additionally an outer membrane.

The semipermeable bacterial membrane functions as a natural barrier to the changing environment and sustains a wide variety of essential cellular processes. Therefore, continuous adaptation of a bacterium to changing environmental conditions requires a corresponding adjustment of the lipid composition. This especially is needed to cope with compounds that are potentially harmful for the integrity of the membrane. Cationic antimicrobial peptides (CAMP) can be found in many organisms exposed to bacterial attacks as part of the intrinsic defense system. These peptides have been shown to directly interact with the negatively charged bacterial membrane as an antibacterial target. A comparable mode of interaction was also suggested for various cationic antibiotics (Zasloff, 2002; Peschel and Sahl, 2006).

Bacteria adjust their membrane lipid composition by modifying the types of fatty acids that are produced *via* various biosynthetic pathways or alternatively by altering the structures of pre-existing membrane lipids (Zhang and Rock, 2008). A major class of membrane lipids are the phospholipids which are glycerolipids that contain two fatty acid chains. These phospholipid acyl chains are mainly responsible for the fluidity of the membrane, which in turn influences many crucial membrane associated functions, such as the passive permeability for hydrophobic molecules, active solute transport and protein-protein membrane localized sterols contributes to membrane fluidity. Moreover, formation of cyclopropane fatty acids in the stationary growth phase renders the bacterial membrane more resistant to acidic stress (Chang and Cronan, 1999).

The polar head group composition of phospholipids in the membrane is tightly controlled. The relevance of this bacterial lipid homeostasis was clearly demonstrated by the analysis of phenotypes of bacterial mutants which are deficient in the related lipid biosynthesis genes. It was shown that the failure of membrane proteins to correctly fold in mutants with altered phospholipid composition gives rise to defects in solute transport, electron transport, the initiation of DNA replication and cell division (Tsatskis *et al.*, 2005; Zhang *et al.*, 2005; Xie *et al.*, 2006).

## 1.2 Structure and Function of Phospholipids

Phospholipids contain a diacylglycerol and a phosphate group which can be modified with other polar groups, for instance ethanolamine or glycerol. The fatty acid residues of phospholipids are hydrophobic, whereas the head group is hydrophilic. This amphipathic character of phospholipids in combination with its almost linear structure is responsible for the formation of a lipid bilayer in an aqueous environment. The cytoplasmic membrane of all living organisms including all Gram-negative and Gram-positive bacteria is composed of this typical lipid bilayer. In contrast, the outer membrane of Gram-negative bacteria contains an asymmetric bilayer which is composed of various phospholipids in the inner leaflet whereas the outer leaflet contains lipopolysaccharides.

The most abundant phospholipids of *Escherichia coli* and *Pseudomonas aeruginosa* are phosphatidylethanolamine (PE) (70 - 80 %), phosphatidylglycerol (PG) (10 - 25 %) and diphosphatidylglycerol (DPG) (5 %) (Hancock and Meadow, 1969; Huijbregts *et al.*, 2000) (Fig. 1).

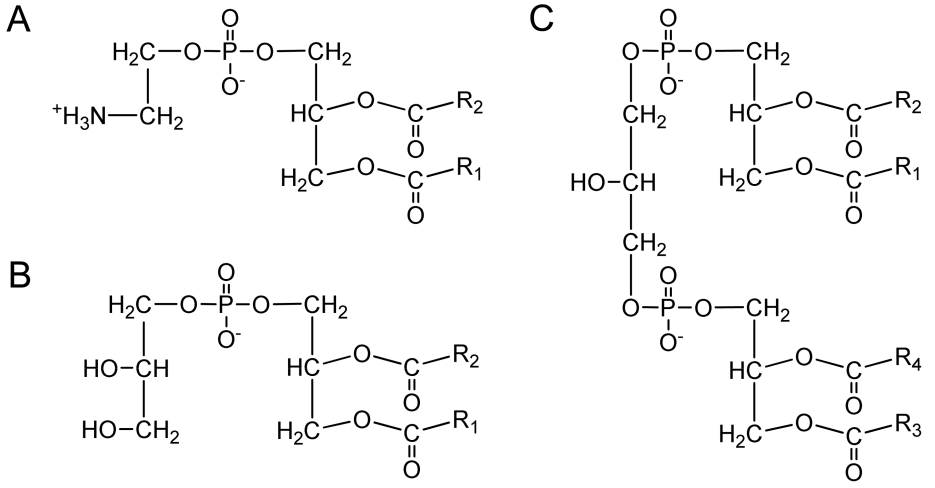



**Figure 1: Structures of phospholipids: phosphatidylethanolamine (*A*), phosphatidylglycerol (*B*) and diphosphatidylglycerol (*C*).**
The phosphate group, the glycerol backbone and the two fatty acid residues are the major constituents of phospholipids. Variations of the head group moiety are shown: *A,* phosphatidylethanolamine; *B,* phosphatidylglycerol; *C,* diphosphatidylglycerol. $R_1$ - $R_4$: fatty acid side chains.

Due to the protonated amino group and the presence of a phosphate group PE is a zwitter ionic molecule under physiological conditions. PG and DPG as negatively charged phospholipids are responsible for the overall net negative charge of the bacterial membrane (Huijbregts *et al.*, 2000).

The primary role of phospholipids is the formation of a semipermeable lipid bilayer. However, they are also involved in several other processes. PE was found essential for the proper folding of the membrane proteins lactose permease LacY, phenylalanine permease PheP and γ-aminobutyrate permease GabP from *E. coli* (Wang *et al.*, 2002; Zhang *et al.*, 2003; Zhang *et al.*, 2005; Xie *et al.*, 2006). In mutant strains lacking PE biosynthesis the cytosolic N-terminal half of LacY was shown to be exposed to the periplasmic site of the bacterial membrane. This change in membrane protein topology impedes the cellular import of lactose (Wang *et al.*, 2002; Xie *et al.*, 2006). Once proper folding of LacY was achieved, PE was dispensable for the active transport of lactose, indicating a functional role of PE as a so called “lipid chaperone” (Bogdanov and Dowhan, 1999; Xie *et al.*, 2006).

The translocation of outer membrane porins PhoE and OmpA and even the assembly of a functional photosynthetic apparatus in *Synechocystis sp.* PCC6803 failed in cells lacking PG (de Vrije *et al.*, 1988; Kusters *et al.*, 1991; Hagio *et al.*, 2000; Sato *et al.*, 2000). Furthermore, PG and DPG are required for initiation of DNA replication by

recruitment of the peripheral membrane protein DnaA to the negative charge surface of the membrane by electrostatic interaction (Matsumoto, 2001). Membranes lacking DPG were found more sensitive to the DNA gyrase inhibitor novobiocin and to other antibiotics (Tropp *et al.*, 1995; Suzuki *et al.*, 2002). The three-fold methylated PE derivative phosphatidylcholine (PC), which is present in about 10 % of all bacteria, was shown to be essential in *Legionella pneumophila* for the attachment to macrophages, the formation of a type IV secretion system and for the transition into a state of higher virulence (Sohlenkamp *et al.*, 2003; Conover *et al.*, 2008).

The functional role of phosphatidylinositol which is found in low abundance in bacteria is not clear. However, phosphatidylinositol formation was shown to be essential for the survival of *Mycobacterium tuberculosis* in macrophages (Jackson *et al.*, 2000).

It becomes clear, that phospholipids play an important role in diverse functions of the bacterial cell.

## 1.3 Phospholipid Biosynthesis

The biosynthesis of phospholipids proceeds *via* a common biosynthetic pathway. Most of the required enzymes can be found conserved in prokaryotes as well as in eukaryotes. Phospholipid synthesis is located at the inner leaflet of the cytoplasmic membrane (Huijbregts *et al.*, 2000; Cronan, 2003).

In all organisms glycerol-3-phosphate is the common precursor of phospholipid biosynthesis (Fig. 2). Glycerol-3-phosphate is either directly synthesized by the phosphorylation of glycerol by glycerol kinase or by glycerol-3-phosphate dehydrogenase *via* the NADH-dependent reduction of dihydroxyacetone phosphate. In a next step the glycerol-3-phosphate acyltransferase PlsB catalyzes the formation of 1-acyl-glycerol-3-phosphate *via* transfer of the acyl group from acyl-CoA or acyl-ACP (acyl carrier protein). Alternatively, 1-acyl-glycerol-3-phosphate is synthesized by conversion of acyl-ACP to acyl-phosphate which is then used for acylation of glycerol-3-phosphate by the glycerol-3-phosphate acyltransferase PlsY (Lu *et al.*, 2006). The second acyl group is added by the 1-acyl-glycerol-3-phosphate acyltransferase to synthesize phosphatidic acid (Frentzen *et al.*, 1983). Acyl-ACP is synthesized in the fatty acid biosynthesis pathway by condensation of acetyl-ACP and

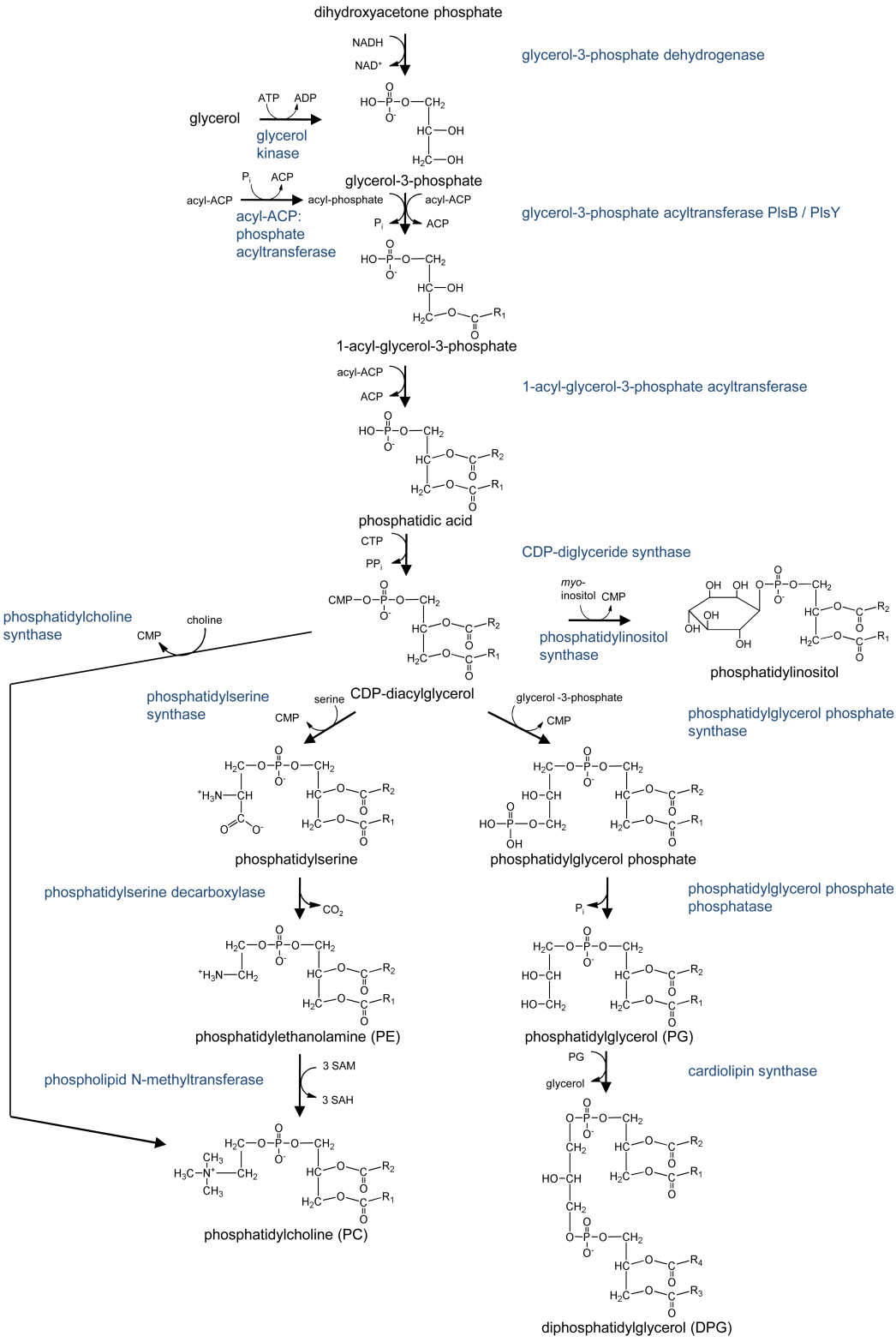



**Figure 2: Overview of the biosynthesis of phospholipids according to Geiger *et al.* (2010).**
All phospholipids are synthesized based on glycerol-3-phosphate. CDP-diacylglycerol is the branching point for the synthesis of the anionic phospholipids PG and DPG and for the zwitter ionic phospholipids PE and PC. Enzymes are shown in *blue*. $R_1$ – $R_4$: fatty acid side chains. ACP = acyl carrier protein. CMP = cytidine monophosphate. CDP = cytidine diophosphate. CTP = cytidine triphosphate. $P_i$ = inorganic phosphate, $PP_i$ = inorganic pyrophosphate. SAM = S-adenosyl-L-methionine. SAH = S-adenosylhomocysteine.

malonyl-ACP and the repeated cycle of reactions involving reduction, dehydration and reduction of carbon-carbon bonds and again the condensation with malonyl-ACP (Rock and Cronan, 1996).

Subsequently, phosphatidic acid is converted to the central phospholipid intermediate cytidine diphosphate diacylglycerol (CDP-diacylglycerol) by the reaction of the CDP-diglyceride synthase (Raetz, 1978). CDP-diacylglycerol is the precursor for formation of PG, DPG, PE, PC and other phospholipids like phosphatidylinositol.

For synthesis of PE and PC, first CDP-diacylglycerol is condensed with serine to form phosphatidylserine. This reaction is catalyzed by the phosphatidylserine synthase. In a second step, catalyzed by the phosphatidylserine decarboxylase, phosphatidylserine is decarboxylated to yield PE. Subsequently, PE can be converted into PC in the "methylation pathway" by the phospholipid-N-methyltransferase *via* three S-adenosyl-L-methionine dependent methylation steps or alternatively, by the PC synthase in the "nucleotide pathway" by condensation of choline and CDP-diacylglycerol (Sohlenkamp *et al.*, 2003).

The phosphatidylglycerol phosphate synthase catalyzes the transfer of glycerol-3-phosphate to CDP-diacylglycerol producing phosphatidylglycerol phosphate. Thereby cytidine monophosphate (CMP) is released. Phosphatidylglycerol phosphate is further converted to PG by the phosphatidylglycerol phosphate phosphatase (Chang and Kennedy, 1967). Finally, two molecules of PG can be condensed in a transesterification reaction of the cardiolipin synthase to yield DPG (De Siervo and Salton, 1971).

A small number of bacteria, for example *M. tuberculosis* contain phosphatidylinositol. This sugar containing phospholipid is synthesized by the condensation of *myo*-inositol to CDP-diacylglycerol catalyzed by the phosphatidylinositol synthase (Jackson *et al.*, 2000).

## 1.4 Aminoacylated Derivatives of Phosphatidylglycerol

Early investigations of the lipid composition of some bacteria revealed the occurrence of aminoacylated phosphatidylglycerol derivatives. The formation of lysyl-phosphatidylglycerol (L-PG) under low pH conditions in *Staphylococcus aureus* and *Enterococcus faecalis* (formerly known as *Streptococcus faecalis*) was already described in the 60s (Houtsmuller and van Deenen, 1965). Furthermore, in 1962 MacFarlane first described the existence of alanyl-phosphatidylglycerol (A-PG) in *Clostridium welchii* (today known as *C. perfringens*) (MacFarlane, 1962). In *E. faecalis* A-PG and L-PG were found simultaneously, whereas the presence of solely A-PG was described for *Bifidobacterium liberorum* (dos Santos Mota *et al.*, 1970; Exterkate *et al.*, 1971). The formation of L-PG by *P. aeruginosa* strain NCTC 6750 was observed by Kenward *et al.* (1979). Moreover, aminoacylation of PG with arginine, glycine and ornithine, respectively, has been described (Houtsmuller and van Deenen, 1963; Gould and Lennarz, 1967; Kocun, 1970). Interestingly, in addition to L-PG formation in the Gram-positive *Listeria monocytogenes* also the modification of DPG with lysine was observed (Fischer and Leopold, 1999). The most abundant aminoacylated PG derivatives L-PG and A-PG are illustrated in Figure 3.

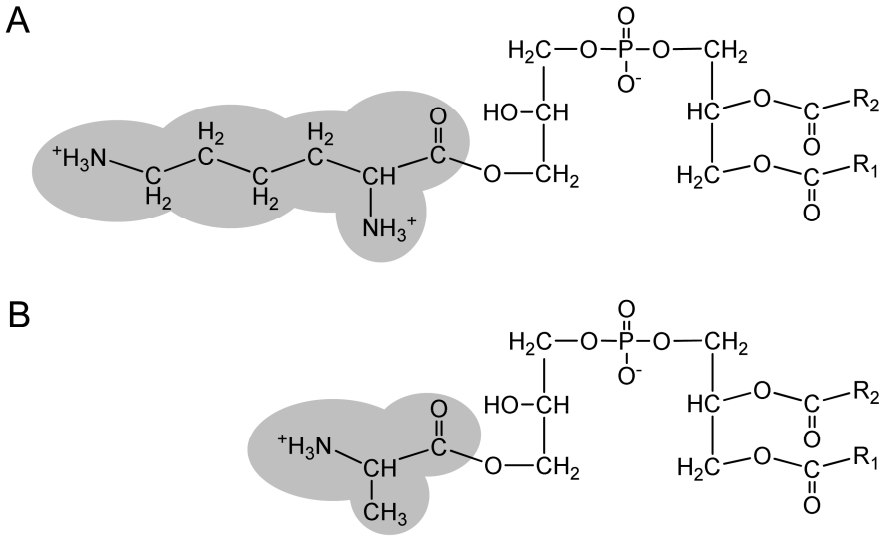



**Figure 3: Structures of lysyl-phosphatidylglycerol (*A*) and alanyl-phosphatidylglycerol (*B*).**
Lysyl-phosphatidylglycerol and alanyl-phosphatidylglycerol are derivatives of PG. The aminoacyl residues of the two molecules are highlighted in *grey*. $R_1$ and $R_2$: fatty acid side chains.

Aminoacyl-PGs are either zwitter ionic phospholipids or alternatively they possess a positive net charge, dependent on the utilized amino acid residue (MacFarlane, 1962; Houtsmuller and van Deenen, 1963; Fischer and Leopold, 1999; Sohlenkamp *et al.*, 2007; Roy and Ibba, 2008b). Therefore, aminoacylation of PG is a process by which the negative net charge of the bacterial membrane is reduced. However, to date it is not clear whether lysinylation of PG, alanylation of PG but also the modification with other amino acids have a comparable effect on the physiology of the organism.

### 1.4.1 Aminoacyl-Phosphatidylglycerol Synthase MprF from *Staphylococcus aureus*

In transposon mutagenesis studies with Gram-positive *Staphylococcus xylosus,* a mutant with an increased sensitivity to the lantibiotic gallidermin was observed. The insertion of the transposon had inactivated an open reading frame (ORF) of 2'525 bp. The corresponding gene of the opportunistic pathogenic Gram-positive bacterium *S. aureus* encodes a putative membrane protein of 841 amino acids which showed no similarities to known proteins. The mutant strain of *S. aureus* was found incapable of L-PG synthesis and was thereby rendered sensitive to cationic defensins when compared with the wild type strain. Due to the increased sensitivity to a variety of CAMPs this enzyme was named MprF (multiple peptide resistance factor) (Peschel *et al.*, 2001). It was concluded that the biosynthesis of L-PG is an important determinant for the pathogenicity of *S. aureus* since the reduction of the overall net anionic charge of the bacterial membrane led to reduced binding to the membrane and cellular permeability for defensins. A repulsion of cationic antimicrobial compounds as a 'defense mechanism' was proposed (Peschel *et al.*, 2001) (Fig. 4).

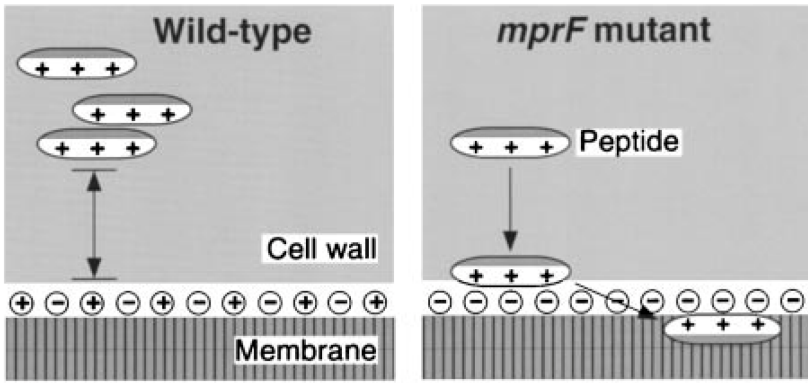



**Figure 4: Putative mode of action of the resistance mechanism to antimicrobial peptides of *S. aureus* (Peschel *et al.*, 2001).**
The cell wall and membrane of *S. aureus* are shown. The hydrophobic and positively charged portions of CAMPs are indicated in *grey* and *white*, respectively. In wild type bacteria a high amount of PG is esterified with lysine residues. Positively charged L-PG results in a reduced binding capacity of the cell envelope for CAMPs. In the *mprF* mutant cell lacking L-PG CAMPs accumulate in the membrane composed mainly of negatively charged lipids (Peschel *et al.*, 2001).

The positively charged L-PG results into a reduced binding capacity of the cell envelope for CAMPs, whereas in the *mprF* mutant cell lacking L-PG CAMPs accumulate in the membrane composed mainly of negatively charged lipids (Peschel *et al.*, 2001). The accumulation of CAMPs in the membrane was proposed to result in the leakage of intracellular content and subsequently to cell death (Zasloff, 2002).

### 1.4.2 Orthologous MprF Proteins

Proteins homologous to MprF from *S. aureus* were found conserved in many pathogenic as well as in non-pathogenic bacteria. They are distributed over almost all phyla of bacteria and were also observed in archaea of the genus *Methanosarcina*.
During the last decade, enzymes responsible for L-PG synthesis were also discovered and investigated for *Bacillus subtilis* (YfiW-YfiX), *Sinorhizobium medicae* WSM419 (LpiA), *Listeria monocytogenes* (Lmo1695), *Rhizobium tropici* CIAT899 (LpiA), *Mycobacterium tuberculosis* (LysX), *Agrobacterium tumefaciens* (NP_355467) and *Bacillus anthracis* (Ba1486) (Staubitz and Peschel, 2002; Reeve *et al.*, 2006; Thedieck *et al.*, 2006; Sohlenkamp *et al.*, 2007; Maloney *et al.*, 2009; Roy and Ibba, 2009; Samant *et al.*, 2009).

Studies with the orthologous *mprF* genes from *L. monocytogenes, R. tropici, M. tuberculosis* and *B. anthracis* revealed an analogous resistance mechanism to CAMPs as described for *S. aureus* (Thedieck *et al.*, 2006; Sohlenkamp *et al.*, 2007; Maloney *et al.*, 2009; Samant *et al.*, 2009). Furthermore, a *S. medicae* mutant which lacks the *mprF* homolog gene *lpiA* (*low-pH inducible gene A*) was compromised in its ability to survive under acidic conditions (Reeve *et al.*, 2006).

Most organisms only contain a single gene encoding an MprF homologous protein. However, in the Gram-positive bacterium *C. perfringens* SM101 two homologous genes were identified, one coding for a lysyl-phosphatidylglycerol synthase (L-PGS) and an additional one coding for an alanyl-phosphatidylglycerol synthase (A-PGS) (Roy and Ibba, 2008b). Formation of A-PG and L-PG in *C. perfringens* was speculated as a mechanism for fine-tuning of the biophysical properties of the bacterial membrane (Roy and Ibba, 2008b).

Based on computational analysis for characterized L-PG and A-PG synthesizing enzymes a two domain architecture consisting of an N-terminal transmembrane domain and an additional C-terminal domain was proposed. The N-terminal domains of these enzymes from various organisms are highly variable in size and share only an amino acid sequence identity of approximately 15 %. In *C. perfringens* A-PGS, this domain consists of 228 amino acid residues, whereas the N-terminal domain of L-PGS from *B. anthracis* contains 527 amino acid residues. In contrast to this the C-terminal domains of aminoacyl-phosphatidylglycerol synthases (aa-PGS) were found highly conserved with an amino acid sequence identity of approximately 30 %.

Interestingly, *M. tuberculosis lysX* encodes an L-PGS as a fusion to a lysyl-tRNA synthetase which is indispensable for L-PG synthesis (Maloney *et al.*, 2009).

From these theoretical findings it was concluded, that the C-terminal domain might be essential for synthesis of aminoacyl-PG (aa-PG).

### 1.4.3 Mechanism of aa-PG Synthesis

The enzymatic reaction mechanism of the synthesis of aa-PG is so far unknown. Based on the high degree of amino acid sequence conservation for the C-terminal domain it was concluded, that this part of the molecule carries the key amino acid residues responsible for A-PG or L-PG catalysis.

After treatment of *S. aureus* infections with daptomycin several daptomycin-resistant clinical isolates with several mutations in the *mprF* gene were observed. Daptomycin is a cyclic lipoprotein antibiotic which acts as CAMP in combination with $Ca^{2+}$ (Straus and Hancock, 2006; Baltz, 2009). The resistant strains carried *mprF* genes encoding the following amino acid exchanges: E44A, S295L, P314L, T345A, T345I, I420N, I420S, I420T, I506M, and L826F (*S. aureus numbering*). These amino acid residues are not conserved in other MprF homolog proteins and most prominently located in the N-terminal transmembrane domain (Friedman *et al.*, 2006; Julian *et al.*, 2007; Kosowska-Shick *et al.*, 2009).

Several conserved amino acid residues of the C-terminal domain (proposed to contain the active site) of *S. aureus* MprF were exchanged (D546A, K547A, K621A, E624A, E685A, D731A, R734A, K806; *S. aureus numbering*) (Ernst *et al.*, 2009). Amino acid exchanges K547A, K621A, E624A, D731A, R734A, and K806A lead to completely abrogate L-PG synthesis under *in vivo* conditions (Ernst *et al.*, 2009). However, these analyses did not provide further insights into the related enzymatic mechanism.

### 1.4.4 Substrate Recognition

The most prominent role of tRNAs is the transfer of an activated amino acid residue to the ribosome during mRNA translation in protein synthesis. However, other processes than protein synthesis in which aminoacylated tRNAs serve as amino acid donor are known. These processes include peptidoglycan crosslinking, synthesis of antibiotics and tetrapyrrole biosynthesis (Matsuhashi *et al.*, 1965; Yamato *et al.*, 1986; Smith *et al.*, 1992). Initial evidence for a tRNA-dependent transfer of the aminoacyl-moiety to PG was obtained in early studies of Gould *et al.* (1968) and Nesbitt *et al.* (1968). It was shown that MprF from *S. aureus* uses $tRNA^{Lys}$ from different bacteria. Furthermore,

$tRNA^{Lys}$ aminoacylated with the lysine analog S-β-aminoethylcysteine was employed as substrate by MprF and resulted in the synthesis of S-β-aminoethylcysteinyl-PG. In contrast, S-β-aminoethylcysteinyl-$tRNA^{Cys}$ did not serve as substrate, indicating that the aa-PGS enzymes might possess a high degree of specificity for their cognate tRNAs (Nesbitt and Lennarz, 1968). In addition, Gould and co-workers tested several modified Ala-tRNA derivatives as substrate for A-PGS from the Gram-positive bacterium *C. perfringens*. All substrate analogs containing modified amino acid moieties (N-acetylalanyl-$tRNA^{Ala}$, lactyl-$tRNA^{Ala}$ and phenylalanyl-$tRNA^{Ala}$) did not sustain any A-PGS activity which clearly indicates that this part of the aminoacylated tRNA substrate is recognized with a high degree of specificity. Furthermore, it was shown, that also the $tRNA^{Ala}$ part of the molecule is relevant for substrate recognition, since Ala-$tRNA^{Cys}$ was not tolerated as an A-PGS substrate (Gould *et al.*, 1968). Roy and Ibba (2008) were able to show that the overall integrity of $tRNA^{Ala}$ is not a prerequisite for *C. perfringens* A-PGS catalysis (Roy and Ibba, 2008b).

Only recently, it was shown that some aa-PG enzymes contain expanded amino acid specificities. Under *in vitro* conditions for the *Enterococcus faecium* aa-PGS a broad specificity for lysine, arginine and alanine was shown, whereas for the orthologous *B. subtilis* protein a primary specificity for lysine, in parallel with a relaxed specificity allowing for A-PG synthesis was observed (Roy and Ibba, 2009). However, for the A-PGS from *C. perfringens* and the L-PGS from *A. tumefaciens* strict amino acid specificity for alanine and lysine, respectively, was demonstrated.

Concerning the polar head group of PG it was shown that only a 2'deoxy-glycerol instead of a glycerol head group was tolerated as substrate (Lennarz *et al.*, 1967). Specific recognition of this part of the molecule was concluded.

These analyses revealed first insights in the recognition of the tRNA and the PG substrate of aa-PGS enzymes.

### 1.4.5 Truncated MprF Homologs with Catalytic Activity

Ernst *et al.* (2009) described the analysis of a truncated MprF variant under *in vivo* conditions. Deletion of 8 of the initial transmembrane helices out of the postulated 14 transmembrane helices comprising the N-terminal transmembrane domain still resulted in L-PGS activity for the enzyme from *S. aureus* (Ernst *et al.*, 2009). Nonetheless, the *S. aureus* strain producing this truncated MprF variant was shown to be susceptible to daptomycin analogous as the *mprF* mutant strain. Based on this observation it was speculated that the deleted N-terminal transmembrane helices are required for the efficient translocation of L-PG to the outer leaflet of the cytoplasmic membrane. A putative flippase activity in the 8 N-terminal transmembrane helices was proposed (Ernst *et al.*, 2009). Based on these results in combination with the tRNA-dependent reaction mechanism (chapter 1.4.4) the following model was proposed (Fig. 5).

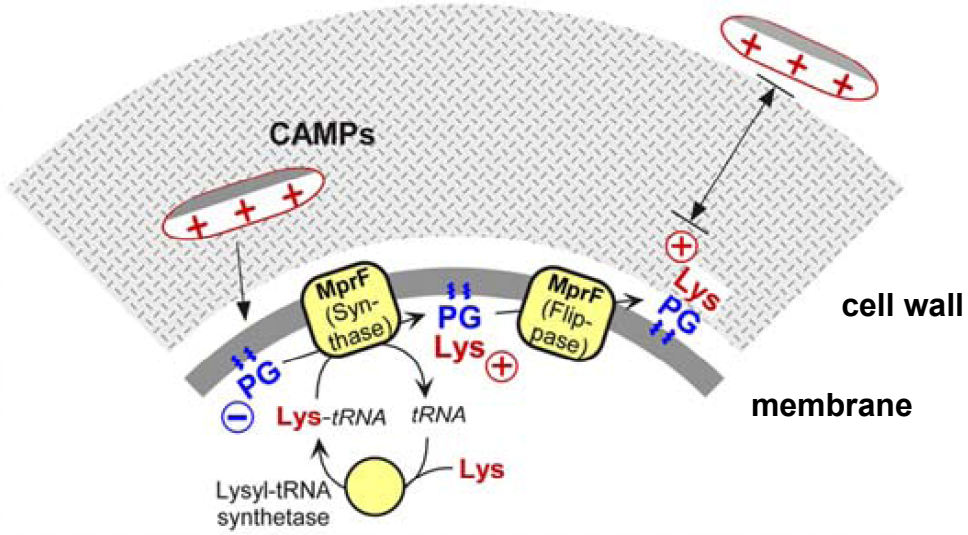



**Figure 5: Proposed model of L-PG synthesis by MprF from *S. aureus* and mode of MprF-mediated bacterial CAMP resistance (Ernst *et al.*, 2009).**
The staphylococcal cell wall and membrane are shown. L-PG is synthesized by the transfer of a lysyl-moiety from Lys-tRNA$^{Lys}$ to PG. This reaction is catalyzed by the "synthase domain" of MprF. L-PG is then translocated from the inner leaflet of the cytoplasmic membrane to the outer leaflet by the putative flippase activity located in the 8 N-terminal transmembrane helices. L-PG formation results in a reduction of the overall net negative charge of the membrane surface (Ernst *et al.*, 2009).

In *in vitro* experiments with *B. subtilis* L-PGS and the *C. perfringens* A-PGS it was shown that a mutant protein with a truncation of the complete hydrophobic N-terminal domain still allows for enzymatic activity (Roy and Ibba, 2009). However, these mutant proteins did not sustain detectable lipid modification under *in vivo* conditions (Roy and

Ibba, 2009). These results indicate that most of the N-terminal domain or in the case of *B. subtilis* and *C. perfringens* the complete N-terminal domain is dispensable for aminoacylation of PG. From these experiments one might conclude that the key amino acid residues responsible for catalysis are located in the C-terminal domain.

## 1.5 The MprF Homolog Protein of *Pseudomonas aeruginosa*

### 1.5.1 *Pseudomonas aeruginosa*: a Brief Introduction

*Pseudomonas aeruginosa* is a metabolically versatile Gram-negative bacterium which is well known for its successful adaptation to several environmental niches (Stover *et al.*, 2000). *P. aeruginosa* is also an opportunistic pathogen responsible for a variety of nosocomial infections and burn-related sepsis (van Delden and Iglewski, 1998). It is the dominant pathogen of cystic fibrosis infections after antibiotics treatment (van Delden and Iglewski, 1998; Koch and Hoiby, 2000; Lyczak *et al.*, 2002). During various infectious and non-infectious states, *P. aeruginosa* faces changing environmental conditions including highly variable pH values. Natural aquatic and terrestic habitats of *P. aeruginosa* span a pH range from 4.5 to 9.5. Moreover, under conditions of cystic fibrosis the airway surface liquid of the lung was found acidified to pH < 6.5 due to defective bicarbonate ion transport (Coakley *et al.*, 2003). This pH alteration was proposed to contribute to cystic fibrosis pathogenesis. Moreover, a typical inflammatory response includes an acidification of the corresponding site by the production of acids (Simmen *et al.*, 1994).

Little is known about the alteration of membrane charge and membrane composition in Gram-negative bacteria including *P. aeruginosa* in response to changing environmental conditions. In earlier studies modification of the overall phospholipid composition of *P. aeruginosa* was described as a result of the stationary phase and starvation (Hancock and Meadow, 1969; Kenward *et al.*, 1979). Conrad and Gilleland (1981) have observed the specific reduction of the phospholipid content of PG and PE for a polymyxin resistant *P. aeruginosa* strain. Furthermore, formation of L-PG by *P. aeruginosa* strain NCTC 6750 was observed under glucose and magnesium depletion by Kenward *et al.* (1979). In *P. aeruginosa* isolates from cystic fibrosis patients modification of LPS

component lipid A by addition of aminoarabinose as well as palmiate and retention of 3-hydroxydecanoate resulted into increased resistance to CAMPs and β-lactam antibiotics (Ernst *et al.*, 2007).

*P. aeruginosa* exhibits high resistance rates to several antimicrobial agents which make it exceedingly difficult to treat infections. This general resistance was ascribed to a combination of different factors. *P. aeruginosa* is intrinsically resistant to many structurally unrelated antimicrobial agents as a result of the low permeability of the outer membrane and due to the presence of highly efficient efflux pump systems (Livermore, 1984; Poole, 2001; Mesaros *et al.*, 2007). Furthermore, *P. aeruginosa* possesses several resistance genes against antibiotics and the formation of *P. aeruginosa* biofilms creates an additional barrier hampering antibiotic treatment (Stewart and Costerton, 2001; Lambert, 2002).

The understanding of underlying resistance mechanisms is of prime importance to find new targets for the efficient treatment of *P. aeruginosa* infections.

### 1.5.2 ORF PA0920 from *Pseudomonas aeruginosa* Encodes an MprF Homolog Protein

The gene product of ORF PA0920 from *P. aeruginosa* PAO1 shares 22 % sequence identity with the MprF protein from *S. aureus*. Based on this theoretical analysis a similar function was proposed. The overproduction of ORF PA0920 in *E. coli* resulted in the synthesis of a new lipid (Fig. 6) (Lorenzo, 2006; Klein, 2007).

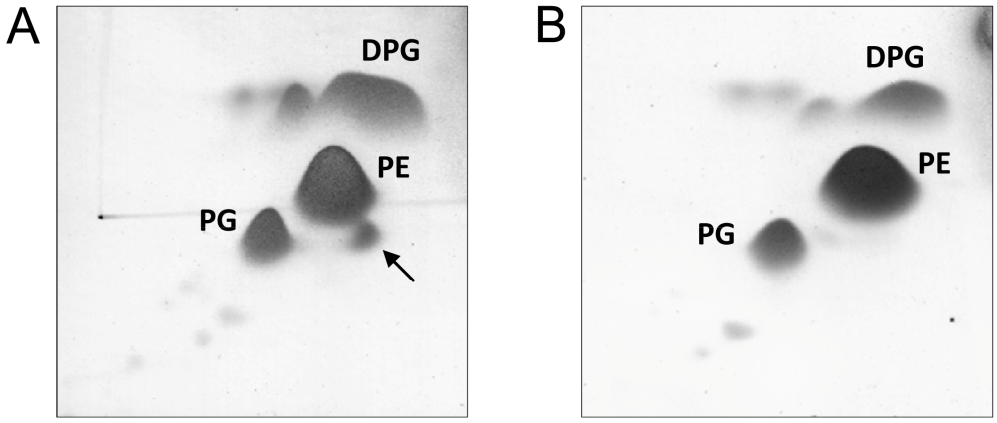



**Figure 6: Detection of membrane lipids of A-PGS overproducing *E. coli* (Klein, 2007).**
An analysis of lipid composition of *E. coli* cultivated in LB medium by two-dimensional thin layer chromatography (2D-TLC) and spraying with 5 % (w/v) molybdatophosphoric acid is shown. *E. coli* TOP10 cells expressing ORF PA0920 from *P. aeruginosa* (*A*) results in the synthesis of a new lipid (indicated by an *arrow*) which is absent from *E. coli* control strain (*B*). PE = phosphatidylethanolamine, PG = phosphatidylglycerol, DPG = diphosphatidylglycerol.

This new lipid was stained positive with molybdenum blue and ninhydrin indicating an amino group containing phospholipid (Lorenzo, 2006; Klein, 2007). In mass spectrometry analysis this lipid was clearly identified as A-PG (Piekarski, 2007). Therefore, the PA0920 protein was termed to A-PGS. The overproduction of A-PGS in *E. coli* TOP10 resulted in the accumulation of approximately 5 % A-PG when compared to the total lipid content. Furthermore, recombinantly overproduced A-PGS was found localized in the membrane fraction of *E. coli* and was solubilized using Triton X-100 (Klein, 2007). Subsequently, solubilized A-PGS was subjected to an *in vitro* assay resulting in A-PG formation. RNase pre-treatment of such experiments abolished A-PG formation which might also indicate a tRNA-dependent catalysis of A-PGS from *P. aeruginosa*.

## 1.6 Aim of this Study

Aminoacyl-phosphatidylglycerol synthases (aa-PGS) catalyze the tRNA-dependent modification of the phospholipid phosphatidylglycerol (PG) to aminoacyl-PG and render bacteria resistant to CAMPs. Consequently, the inhibition of this class of enzymes is a promising strategy to render pathogenic bacteria more susceptible to antibacterial agents. To find a rationale for the development of efficient inhibitor molecules the understanding of the physiological relevance of aa-PGS catalysis and the underlying enzymatic mechanism is of prime importance.

The first objective of this work was to investigate conditions under which the alanyl-phosphatidylglycerol synthase (A-PGS) from *Pseudomonas aeruginosa* is upregulated in the natural host. Furthermore, it was intended to identify physiological conditions under which A-PG synthesis is beneficial for *P. aeruginosa*. For this purpose, phenotypical differences between the *P. aeruginosa* wild type and a deletion mutant strain lacking the A-PGS encoding ORF PA0920 had to be determined by using standard microbiological methods and also by using a phenotype microarray analysis system.

A second objective was to establish a minimal A-PGS catalytic fragment. Once identified, the truncated A-PGS variant should be recombinantly produced, purified and biochemically characterized. For analysis of A-PGS activity *in vivo* and *in vitro* assays had to be developed.

Finally, the catalytic reaction mechanism was of central interest. For its elucidation several mutant variants of A-PGS had to be produced by site directed mutagenesis and subsequently biochemically characterized. Chemical modification experiments should complete the picture. Determinants of the PG and the tRNA substrate important for recognition and catalysis had to be elucidated. Besides this, the topology of the C-terminus of the membrane protein had to be analyzed.

Obtained data should be integrated to a biochemical model of A-PGS function and regulation for the design of future inhibition strategies.

# 2 MATERIALS AND METHODS

## 2.1 Instruments, Chemicals and Materials

### 2.1.1 Instruments

| | | |
|---|---|---|
| agarose gel electrophoresis | Agagel | Biometra |
| agarose gel documentation | GelDoc | BioRad |
| autoclave | LVSA 50/70 | Zirbus |
| blotting equipment | Semidry-Blot Trans-Blot®SD | BioRad |
| centrifuges | Centrifuge 5804 | Eppendorf |
| | Minispin | Eppendorf |
| | RC 5B Plus | Sorvall |
| | L7-65 Ultracentrifuge | Beckman |
| DNA sequencing | ABI Prism™ 310 Genetic Analyzer | Applied Biosystems |
| FPLC | ÄKTApurifier™ | GE Healthcare |
| French® Press | French® Pressure Cell | SLM Aminco |
| | French® Pressure Cell Press | Polytec |
| luminescence (fluorescence) spectrometer | LS50B | Perkin Elmer |
| lyophilization | Lyovac GT2 | Amsco/Finn-Aqua |
| mass spectrometer | QTOF 2 mass spectrometer | Micromass |
| pH determination | pH-Meter CG 842 | Schott |
| photometer | Ultrospec 2000 | Amersham Pharmacia |
| power supply electrophorese | PowerPac 300 | Biorad |
| scales | SBA 52 | Scaltec |
| | BP61S | Sartorius |
| scintillation counting | TriCarb 2900 TR | Perkin Elmer |
| SDS-PAGE | Mini Protean III | BioRad |
| shaker | 305/3020 | GFL |
| | TR-150 | Infors AG |
| | Bench Top Shaker, TR | Infors AG HT |
| sonication | UW 2070 | Bandelin |
| | Sonotrode MS73 | Bandelin |
| Speed vac® | SPD101B | Savant |
| thermocycler | Tpersonal | Biometra |
| | Tgradient | Biometra |
| thermomixer | Thermomixer 5436 | Eppendorf |
| vortex | Vortex-Genie 2 | Scientific Industries |
| water purification | Milli-Q System | Millipore |
| X-ray developer | Optimax® | Protec® |

### 2.1.2 Chemicals and Materials

| | | |
|---|---|---|
| antibodies | anti-His (murine) | GE Healthcare |
| | anti-mouse HRP-conjugate (Fc-specific) | Pierce |
| | anti-*myc*-antibody (murine) | Invitrogen |
| blotting materials | Gel Blotting Papers | Roth |
| | Roti-PVDF-membrane | Roth |
| chemicals | Bradford reagent | Sigma-Aldrich |
| | GelStar® Nucleic Acid Gel Stain | Cambrex |
| | Molybdatophosphoric acid | Merck |
| | phosphatidylglycerol (L-α-phosphatidyl-DL-glycerol sodium salt from egg yolk lecithin) | Sigma-Aldrich |
| | diphosphatidylglycerol (cardiolipin from bovine heart) | Sigma-Aldrich |
| enzymes | creatin phosphokinase | Sigma-Aldrich |
| | Phusion™ polymerase | Finnzymes |
| | PreScission Protease | GE Healthcare |
| | restriction enzymes | New England Biolabs |
| | Ribonuclease A | Sigma-Aldrich |
| | T4 DNA Ligase | New England Biolabs |
| | *Taq* DNA polymerase | Biotherm |
| kits | QIAquick Gel Extraction Kit | Qiagen |
| | QIAquick PCR Purification Kit | Qiagen |
| | QuikChange® Site-Directed Mutagenesis Kit | Agilent Technologies |
| | SuperSignal® West Pico Chemiluminescence Kit | Pierce |
| molecular weight standards | GeneRuler™ DNA Ladder Mix | Fermentas |
| | Protein Molecular Weight Marker | Fermentas |
| | PageRuler™ Prestained Protein Ladder | Fermentas |
| | molecular weight marker Kit | Sigma-Aldrich |
| PCR materials | nucleotides (dNTPs, rNTPs) | Fermentas |
| | oligonucleotides | Biomers, MWG Biotech, Integrated DNA Technologies |
| radioactively labeled molecules | [U-$^{14}$C]-L-alanine, 162 mCi mmol$^{-1}$ | Moravek Biochemicals |
| | [1-$^{14}$C]-L-alanine, 51 mCi mmol$^{-1}$ | Moravek Biochemicals |
| | [2,3-$^{3}$H]-L-alanine, 52 mCi mmol$^{-1}$ | GE Healthcare |
| | [U-$^{14}$C]-L-histidine, 322 mCi mmol$^{-1}$ | GE Healthcare |
| | [U-$^{14}$C]-L-lysine, 312 mCi mmol$^{-1}$ | Moravek Biochemicals |
| scintillation solution | OptiPhase HighSafe 2 | Perkin Elmer |
| thin layer chromatography | DC-plates Alugram® SIL G/UV254 | Macherey-Nagel |
| other materials | Chelating Sepharose FF | GE Healthcare |
| | Glutathione Sepharose 4FF | GE Healthcare |
| | gravity flow column “Poly-Prep”, 0.8 x 4 cm | BioRad |
| | Microcon® Centrifugal Filter Unit | Millipore |
| | Slide-A-Lyzer® MINI dialysis units | Pierce |
| | sterile filter | Millipore, Sartorius |
| | Superdex 75 HR 10/30 | GE Healthcare |
| | MonoQ 5/50 GL | Pharmacia Biotech |

Chemicals and reagents not specifically listed here were purchased from the following manufacturers: Fluka, GE Healthcare, Macherey-Nagel, Merck, Riedel-de Haën, Roche, Roth, and Sigma-Aldrich.

## 2.2 Bacterial Strains, Plasmids and Primers

### 2.2.1 Bacterial Strains

All bacterial strains used in this work are listed in Table 1.

**Table 1: *P. aeruginosa* and *E. coli* strains used in this study.**

| Bacterial strain | Genotype or phenotype | Reference |
|---|---|---|
| *P. aeruginosa* | | |
| PAO1 | Wild type | (Dunn and Holloway, 1971) |
| ADD1976 | PAO1 derivative containing *lac*UV5/*lacI*$^q$-regulated T7 RNA polymerase gene | (Brunschwig and Darzins, 1992) |
| KS11 | PAO1 *attB*::(mini-CTX-*lacZ*) | (Schreiber *et al.*, 2006) |
| PAO-MW20 | PAO1 *rpoS*::*aacC,* Gm$^r$ | (Whiteley *et al.*, 2000) |
| NB170 | PAO1 Δ*relA* Δ*spoT* | (Boes *et al.*, 2008) |
| ΔPA0920 | *P. aeruginosa* ΔPA0920 deletion mutant | (Lorenzo, 2006) |
| $P_{PA0920}$-*lacZ* | *P. aeruginosa* carrying a $P_{PA0920}$-*lacZ* reporter gene fusion integrated at the *attB* site | (Spier, 2007) |
| ΔPA0920compl | ΔPA0920 *attB*::(mini-CTX2-PA0920), complementation of ΔPA0920 *via* integration of the region ranging from 187 bp downstream to 90 bp upstream of the PA0920 region into the *attB* site | This work |
| PAO-SK01 | ΔPA0920 *attB*::(mini-CTX2) | This work |
| PAO-SK02 | PAO1 *attB*::(mini-CTX2) | This work |
| PAO-SK03 | PAO-MW20 carrying a $P_{PA0920}$-*lacZ* reporter gene fusion integrated at the *attB* site | This work |
| PAO-SK04 | NB170 carrying a $P_{PA0920}$-*lacZ* reporter gene fusion integrated at the *attB* site | This work |
| *E. coli* | | |
| DH10B | F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX*74 *deoR recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*) 7697 *galU galK* λ$^-$ *rpsL nupG* | Invitrogen, Darmstadt, Germany |

| Bacterial strain | Genotype or phenotype | Reference |
|---|---|---|
| TOP10 | F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str$^r$) *end*A1 *nup*G | (Grant *et al.*, 1990) |
| BL21 (λDE3) | F$^-$ *ompT gal dcm lon hsdS$_B$*($r_B^-$ $m_B^-$) λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5]) | (Studier *et al.*, 1990) |
| BL21 (DE3)-RIL CodonPlus™ | F$^-$ *ompT hsdS*($r_B^-$ $m_B^-$) *dcm*$^+$ Tet$^r$ *gal* λ(DE3) *endA Hte*[*argU ileY leuW*], Cam$^r$ | Stratagene, Santa Clara, CA, USA |
| Rosetta (DE3) pLysS | F$^-$ *ompT hsdS*$_B$($r_B^-$ $m_B^-$) *gal dcm* (DE3) pLysSRARE, Cam$^r$ | Novagen, Darmstadt, Germany |
| Rosetta 2 (DE3) | F$^-$ *ompT hsdS*$_B$($r_B^-$ $m_B^-$) *gal dcm* (DE3) pRARE2, Cam$^r$ | Novagen, Darmstadt, Germany |
| K-12, strain AG1, clone JW2667 | *recA1 endA1 gyrA96 thi-1 hsdR17(rK$^-$ mK$^+$) supE44 relA1*, carrying plasmid pCA24N which encodes for alanyl-tRNA synthetase from *E. coli,* Cam$^r$ | (Kitagawa *et al.*, 2005) |
| K-12, strain AG1, clone JW2498 | *recA1 endA1 gyrA96 thi-1 hsdR17(rK$^-$ mK$^+$) supE44 relA1*, carrying plasmid pCA24N which encodes for histidyl-tRNA synthetase from *E. coli,* Cam$^r$ | (Kitagawa *et al.*, 2005) |
| ST18 | S17 λ-pir Δ*hemA* | (Thoma and Schobert, 2009) |

### 2.2.2 Plasmids

All plasmids used in this work are listed in Table 2.

**Table 2: Plasmids used in this work.**

| Plasmid | Description | Reference |
|---|---|---|
| mini-CTX-*lacZ* | chromosomal integration vector for *P. aeruginosa* carrying a promoterless *lacZ* gene, Tc$^r$ | (Becher and Schweizer, 2000) |
| mini-CTX-$P_{PA0920}$-*lacZ* | mini-CTX-*lacZ* containing a 469 bp fragment of the upstream region of the PA0920 gene cloned into *Xho*I and *Eco*RI sites | (Spier, 2007) |
| mini-CTX2 | chromosomal integration vector for *P. aeruginosa*, Tc$^r$ | (Hoang *et al.*, 2000) |
| pFLP2 | source of FLP recombinase, Ap$^r$ | (Hoang *et al.*, 1998) |
| pUCP20T | shuttle vector *E. coli-P. aeruginosa*, Cb$^r$ | (West *et al.*, 1994) |
| pET22b(+) | expression vector for *E. coli*, $P_{T7}$, *ori*$_{pBR322}$, *lacI*, C-terminal $His_6$-tag, Ap$^r$ | Novagen, Darmstadt, Germany |

| Plasmid | Description | Reference |
|---|---|---|
| pET28b(+) | expression vector for *E. coli*, $P_{T7}$, $ori_{pBR322}$, *lacI*, N-terminal $His_6$-tag, $Kan^r$ | Novagen, Darmstadt, Germany |
| pBAD-His-A | expression vector for *E. coli*, $P_{araBAD}$, $ori_{pBR322}$, N-terminal $His_6$-tag, $Ap^r$ | Invitrogen, Darmstadt, Germany |
| pBAD*myc*-His-A | expression vector for *E. coli*, $P_{araBAD}$, $ori_{pBR322}$, C-terminal $His_6$-tag and myc epitope, $Ap^r$ | Invitrogen, Darmstadt, Germany |
| pGEX-6P-1 | *E. coli* expression vector for N-terminal protein fusion with GST-tag, specific cleavage site for PreScission Protease, $P_{tac}$, $ori_{pBR322}$, $Ap^r$ | GE Healthcare, Munich, Germany |
| pUC18 | expression vector for *E. coli*, $P_{lac}$, $ori_{pBR322}$, 5'-terminal part of the *lacZ* gene encoding the N-terminal fragment of β-galactosidase, $Ap^r$ | (Yanisch-Perron *et al.*, 1985) |
| pUC119 | expression vector for *E. coli*, $P_{lac}$, $ori_{pBR322}$,5'-terminal part of the *lacZ* gene encoding the N-terminal fragment of β-galactosidase, containing the intergenic region of M13 for production of ssDNA, $Ap^r$ | (Vieira and Messing, 1987) |
| pET15bLysS | expression vector for lysyl-tRNA synthetase from *Borrelia burgdorferi*, $P_{T7}$, $ori_{pBR322}$, N-terminal $His_6$-tag, $Ap^r$ | (Ibba *et al.*, 1997) |
| pT7-911 | expression vector for *E. coli* encoding the T7 RNA polymerase, IPTG inducible promoter, N-terminal $His_6$-tag, $Ap^r$ | Kindly provided by Thomas E. Shrader, Albert Einstein College of Medicine, New York |
| pGEX-6P-1/PA0920 ΔAS1-542 | pGEX-6P-1 carrying base pairs 1627-2643 of the ORF PA0920 from *P. aeruginosa* in *Bam*HI-*Xho*I restriction sites, encoding amino acid residues 543-881 of A-PGS as GST-fusion protein, $Ap^r$ | (Tiefenau, 2007) |
| pGEX-6P-1/*mprF* ΔAS1-514 | pGEX-6P-1 carrying base pairs 1543-2520 of the *mprF* gene from *Staphylococcus aureus* in *Bam*HI-*Xho*I restriction sites, encoding amino acid residues 515-840 of MprF as GST-fusion protein, $Ap^r$ | (Tiefenau, 2007) |
| pGEX-6P-1/*lin1803* ΔAS1-511 | pGEX-6P-1 carrying base pairs 1534-2595 of the *Listeria innocua* ORF *lin1803* in *Bam*HI-*Xho*I restriction sites, encoding amino acid residues 512-865 of Lin1803 as GST-fusion protein, $Ap^r$ | (Tiefenau, 2007) |
| pGEX-6P-1/lmo1695 ΔAS1-510 | pGEX-6P-1 carrying base pairs 1531-2595 of the *Listeria monocytogenes* ORF lmo1695 in *Bam*HI-*Xho*I restriction sites, encoding amino acid residues 511-865 of Lmo1695 as GST-fusion protein, $Ap^r$ | (Tiefenau, 2007) |
| pGEX-6P-1/PA0920 ΔAS1-542 D579A | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange aspartate 579 to alanine | (Arendt, 2009) |

| Plasmid | Description | Reference |
|---|---|---|
| pGEX-6P-1/PA0920 ΔAS1-542 D579N | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange aspartate 579 to asparagine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 E657D | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange glutamate 657 to aspartate | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 E657Q | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange glutamate 657 to glutamine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 E658D | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange glutamate 658 to aspartate | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 E658Q | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange glutamate 658 to glutamine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 S709A | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange serine 709 to alanine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 S709N | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange serine 709 to asparagine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 D710A | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange aspartate 710 to alanine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 D710N | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange aspartate 710 to asparagine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 E720Q | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange glutamate 720 to glutamine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 S724A | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange serine 724 to alanine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 S724N | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange serine 724 to asparagine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 S763A | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange serine 763 to alanine | (Arendt, 2009) |
| pGEX-6P-1/PA0920 ΔAS1-542 S763N | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange serine 763 to asparagine | (Arendt, 2009) |
| pBAD-His-A/PA0920 | pBAD-His-A containing the PA0920 gene fused to the sequence for N-terminal $His_6$-tag cloned into *Xho*I and *Hin*dIII sites | (Klein, 2007) |
| pBAD*myc*-His-A/ PA0920 | pBAD*myc*-His-A containing PA0920 gene fused to the sequence for C-terminal $His_6$-tag cloned into *Hin*dIII and *Nco*I sites | (Lorenzo, 2006) |

| Plasmid | Description | Reference |
|---|---|---|
| pET22b(+)PA0920 | pET22b(+) carrying PA0920 gene in *Nde*I and *Hin*dIII sites, encoding a C-terminal $His_6$-tag | (Piekarski, 2007) |
| mini-CTX2-PA0920 | chromosomal integration vector containing 187 bp of PA0920 upstream region, the PA0920 gene and 90 bp of downstream region into *Sac*I and *Hin*dIII sites | This work |
| pUCP20T-T7-PA0920-$His_6$ | pUCP20T carrying the PA0920 gene fused to the sequence for C-terminal $His_6$-tag, under $P_{T7}$ into *Bam*HI and *Kpn*I sites | This work |
| pET28b(+)alaRS | pET28b(+) carrying the *P. aeruginosa* ORF PA0903 encoding the alanyl-tRNA synthetase in *Nde*I-*Bam*HI sites | This work |
| pUC18tRNA$^{Ala1}$ | pUC18 containing ORF PA4280.3 (coding for tRNA$^{Ala1}$) from *P. aeruginosa* cloned into *Eco*RI and *Bam*HI sites under control of $P_{T7}$, 3' *Bst*NI restriction site | This work |
| pUC119tRNA$^{Ala2}$ | pUC119 containing ORF PA3133.2 (coding for tRNA$^{Ala2}$) from *P. aeruginosa* cloned into *Eco*RI and *Bam*HI restriction sites under control of $P_{T7}$, 3' *Bst*NI restriction site | This work |
| pGEX-6P-1/ A-$PGS_{543\text{-}855N}$ | pGEX-6P-1/PA0920ΔAS1-542 derivative, amino acid exchange alanine 856 to asparagine and deletion of amino acid residues 857-881 | This work |
| pBAD-His-A/ $PA0920_{1\text{-}855N}$ | pBAD-His-A/PA0920-derivative, amino acid exchange alanine 856 to asparagine and deletion of amino acid residues 857-881 | This work |

### 2.2.3 Oligonucleotides

DNA and RNA oligonucleotides used in this work are listed in Table 3 and 4, respectively. Oligonucleotides were purchased from MWG Biotech AG (Ebersberg, Germany), Biomers (Ulm, Germany) or Integrated DNA Technologies (Coralville, IA, USA). Synthetic RNA oligonucleotides were used for mimicking of the acceptor stem of $tRNA^{Ala}$ and $tRNA^{His}$.

**Table 3: DNA oligonucleotides used in this work.**
Restriction sites are shown in *italics*. Exchanged nucleotides for mutagenesis of *P. aeruginosa* A-PGS or introduced stop-codons are highlighted in *bold font* and *Bst*NI restriction sites are *underlined.*

| Oligonucleotide name | Sequence (5'-3') |
|---|---|
| 1PA0920SacIF | GTAAT*GAGCTC*GCTGAACCATCGCTGAAC |
| 2PA0920HindIIIR | GATTATT*AAGCTT*GGTCGGCTCCAGAGCAG |
| 3pET22b+BamHf | GAT*GGATCC*GTCGGCGATATAGGC |
| 4pET22b+KpnIr | GATAT*GGTACC*GCAGCAGCCAACTC |
| 27alasSNdeIfw | ATAGC*CATATG*AAAAGCGCTGAAATCCG |
| 28alsSBclIrv | ATAGC*TGATCA*TCAGAGCCCTTGCTCGAC |
| alatRNA1fw | *AATTC*TAATACGACTCACTATAGGGGCCATAGCTCAGCTGGGAG AGCGCCTGCTTTGCACGCAGGAGGTCAGGAGTTCGATCCTCCTT GGCTCCACCA*G* |
| alatRNA1rv | *GATCC*TGGTGGAGCCAAGGAGGATCGAACTCCTGACCTCCTGCG TGCAAAGCAGGCGCTCTCCCAGCTGAGCTATGGCCCCTATAGTG AGTCGTATTA*G* |
| alatRNA2fw | *AATTC*TAATACGACTCACTATAGGGGCTATAGCTCAGCTGGGAG AGCGCTTGCATGGCATGCAAGAGGTCGACGGTTCGATCCCGTCT AGCTCCACCA*G* |
| alatRNA2rv | *GATCC*TGGTGGAGCTAGACGGGATCGAACCGTCGACCTCTTGCA TGCCATGCAAGCGCTCTCCCAGCTGAGCTATAGCCCCTATAGTG AGTCGTATTA*G* |
| QCP856NSTOPfw | CTACCTGGCCGTGCCA**AACTA**GGGCTCGACCCGCTGGTG |
| QCP856NSTOPrv | CACCAGCGGGTCGAGCCC**TAGTT**TGGCACGGCCAGGTAG |

**Table 4: RNA oligonucleotides used in this work.**
The tetraloops of microhelices are shown in *italics*. The CCA 3' ends are highlighted in *bold font*.

| Oligonucleotide name | Sequence (5'-3') |
|---|---|
| microhelix A | GGGGCUC*UUCG*GAGCUCCA**CCA** |
| microhelix B | GGGGACG*UUCG*CGUCUCCA**CCA** |
| microhelix C | ACGGCUC*UUCG*GAGCUGUG**CCA** |
| microhelix D | ACGGACG*UUCG*CGUCUGUA**CCA** |
| microhelix E | GUGGCUC*UGCG*GAGCUACA**CCA** |
| microhelix F | GGUGGCUC*UGCG*GAGCCACC**CCA** |

## 2.3 Growth Media and Media Additives

### 2.3.1 Media

#### 2.3.1.1 LB medium

As a standard medium for growth of all bacterial strains, Luria Bertani (LB) medium (Sambrook and Russell, 2001) was used unless indicated otherwise. For solid media, 1.5 % (w/v) agar-agar was added before sterilization.

| | | |
|---|---|---|
| LB medium | tryptone | 10.0 g/l |
| | NaCl | 5.0 g/l |
| | yeast extract | 5.0 g/l |

To generate acidic growth conditions (pH 5.5) LB medium was supplemented with 250 mM 2-(*N*-morpholino)ethanesulfonic acid.

For protein production of HisRS and AlaRS in *E. coli* strains AG1 clone JW2498 and JW2667 LB medium with self inducing reagents for T7 promoters were added to avoid the need for IPTG addition according to Studier (2005).

| | | |
|---|---|---|
| LB medium with self inducing reagents | LB medium | 930 ml |
| | 1 M $MgCl_2$ | 1 ml |
| | 50x 5052 | 20 ml |
| | 20x NPS | 50 ml |
| 50x 5052 | glycerol | 250 g/l |
| | glucose | 25 g/l |
| | α-lactose | 100 g/l |
| 20x NPS, pH 6.7 | $(NH_4)_2SO_4$ | 500 mM |
| | $Na_2HPO_4$ x 7 $H_2O$ | 1 M |
| | $KH_2PO_4$ | 1 M |

20x NPS was autoclaved, whereas 50x 5052 was sterilized using a sterile filter unit (pore width 0.2 µm).

#### 2.3.1.2 AB medium

For the cultivation of *P. aeruginosa* AB medium (Heydorn *et al.*, 2000) and also a modified AB medium was used. For solid AB media, 1.5 % (w/v) agar-agar in water was prepared and autoclaved. After cooling, the other compounds of the medium were added.

| | | |
|---|---|---|
| AB medium | standard A10 | 100 ml/l |
| | 1 M glucose | 20 ml/l |
| | 50 mM $FeSO_4$ x 7 $H_2O$ | 500 µl/l |
| | trace elements | 1 ml/l |
| | 1 M $MgCl_2$ | 1 ml/l |
| | 1 M $CaCl_2$ | 100 µl/l |

All compounds were prepared as stock solutions. Standard A10, magnesium chloride, and calcium chloride were autoclaved, whereas glucose, ferrous (II) sulphate and trace elements were sterilized using a sterile filter unit (pore width 0.2 µm).

| | | |
|---|---|---|
| standard A10 | $(NH_4)_2SO_4$ | 20 g/l |
| | $Na_2HPO_4$ x 7 $H_2O$ | 90 g/l |
| | $KH_2PO_4$ | 30 g/l |
| trace elements | $CaSO_4$ x 2 $H_2O$ | 200 mg/l |
| | $FeSO_4$ x 7 $H_2O$ | 200 mg/l |
| | $MnSO_4$ x $H_2O$ | 20 mg/l |
| | $CuSO_4$ x 5 $H_2O$ | 20 mg/l |
| | $ZnSO_4$ x 5 $H_2O$ | 20 mg/l |
| | $NaMoO_4$ x $H_2O$ | 10 mg/l |
| | $H_3BO_3$ | 5 mg/l |

For cultivation of *P. aeruginosa* under acidic and neutral growth conditions a modified AB medium was used. In this medium the pH value was adjusted by using a modified standard A10 buffer system (Table 5) containing 20 g/l ammonium sulphate. For cultivation under alkaline conditions (pH 9.5) 1 M of N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid was added to the standard A10, subsequently the pH value was precisely titrated with NaOH. In all cases the pH value of the individual media was verified at the beginning and at the end of the cultivation.

**Table 5: pH-modified A10.**
A10 with different pH values were adjusted by the phosphate buffer system.

| pH value A10 | $Na_2HPO_4$ x 7 $H_2O$ | $KH_2PO_4$ | pH value of AB media |
|---|---|---|---|
| 4 | 1.00 mM | 449.00 mM | 4.3 |
| 4.5 | 2.00 mM | 448.00 mM | 4.8 |
| 5 | 20.00 mM | 430.00 mM | 5.3 |
| 5.5 | 50.00 mM | 400.00 mM | 5.8 |
| 6 | 62.50 mM | 387.50 mM | 6.3 |
| 6.5 | 152.00 mM | 313.00 mM | 6.8 |
| 7 | 300.00 mM | 150.00 mM | 7.3 |

### 2.3.2 Additives

Antibiotics and other additives were prepared as concentrated stock solutions, sterilized by filtration (pore width 0.2 µm) and were added to the medium after autoclaving. The following concentrations were used:

| Substance | Solvent | Concentration of stock solution | Final concentration | |
|---|---|---|---|---|
| | | | cultivation of *E. coli* | cultivation of *P. aeruginosa* |
| ampicillin | $dH_2O$ | 100 mg/ml | 100 µg/ml | - |
| carbenicillin | $dH_2O$ | 100 mg/ml | 100 µg/ml | 250 µg/ml |
| chloramphenicol | ethanol 100 % (v/v) | 34 mg/ml | 34 µg/ml | - |
| kanamycin | $dH_2O$ | 30 mg/ml | 30 µg/ml | - |
| tetracycline | ethanol 70 % (v/v) | 10 mg/ml | 12.5 µg/ml | 80 µg/ml |
| IPTG | $dH_2O$ | 100 mM | 50 – 400 µM | 2 mM |
| L-arabinose | $dH_2O$ | 20 % (w/v) | 0.002 – 2 % | - |

## 2.4 Microbiological Techniques

### 2.4.1 Sterilization

All media were vapour sterilized at 121 °C and 1 bar positive pressure for 20 min. Other substances and solutions were either vapour sterilized or if temperature sensitive sterilized by filtration (pore width 0.2 µm).

### 2.4.2 Cultivation of *E. coli* and *P. aeruginosa*

*E. coli* cells for recombinant protein production were grown under vigorous aeration. Pre-cultures were inoculated from glycerol stocks or single colonies from a plate culture in 100 ml of LB medium containing the respective antibiotics and grown overnight at 37 °C and 200 rpm in baffled flasks. After inoculation with pre-culture (1:100), 500 ml cultures were grown at 37 °C until induction of protein production with indicated amounts of IPTG or L-arabinose. Cultures were then incubated at 17 °C, 30 °C or 37 °C for 3 - 5 h.
*P. aeruginosa* pre-cultures were inoculated from single colonies from a plate culture in 100 ml of LB or AB media containing the respective antibiotics and grown overnight at 37 °C and 200 rpm in baffled flasks. After inoculation with pre-culture (1:100), 500 ml cultures were grown at 37 °C. Protein production was induced with indicated amounts of IPTG. Cultures were further incubated at 37 °C for 4, 6 or 24 h.

Agar plates were either utilized for plating 50 - 100 µl of a bacterial cell suspension with a Drigalski spatula or for streaking cells with an inoculating loop with liquid culture or with a single colony. Plates were incubated aerobically at 37 °C overnight.

### 2.4.3 Determination of Cell Density

The cell density of liquid cultures was determined by measuring the optical density (OD) at a wave length of 578 nm. For cell densities with an $OD_{578} \geq 0.6$ dilutions with the respective cultivation medium were prepared before measurement. An $OD_{578}$ of 1 corresponds to approximately 1 x $10^9$ cells/ml.

### 2.4.4 Storage of Bacteria

Strains were kept on agar plates at 4 °C for up to four weeks. For long term storage glycerol stocks were prepared. Therefore, an overnight culture was mixed with glycerol to yield a final concentration of 20 % (w/v) and kept at -80 °C.

### 2.4.5 Immunogold-Labeling

*E. coli* TOP10 pBAD*myc*-His-A/PA0920 was induced at an $OD_{578}$ of 0.5 with 0.75 % (w/v) L-arabinose and cultivated for 4 h at 37 °C. Bacteria were fixed with 1 % (v/v) formaldehyde in the growth medium at 4 °C. After washing with cacodylate buffer containing 10 mM glycine samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90, 100 % (v/v)) and embedded in London Resin White (LRW) resin. Ultrathin sections were cut with a diamond knife and collected onto butvar coated nickel grids (300 mesh). Sections were incubated with anti-myc epitope mouse monoclonal $IgG_1$ (1:50 dilution of the stock solution containing 1 mg/ml $IgG_1$ antibodies) for 16 h at 4 °C. After washing with 1x PBS bound antibody was made visible by using goat anti-mouse IgG antibodies coupled to 10 nm gold-nanoparticles (1:200 dilution of the stock solution, incubation for 1 h at RT). After washing with 1x PBS containing 1 % (v/v) Tween 20, additional washing steps with 1x PBS and with distilled water were performed. Then air-dried sections were counterstained with 4 % (w/v) aqueous uranyl acetate for 3 min before examination in a Zeiss transmission electron microscope EM910 at an acceleration voltage of 80 kV and calibrated magnifications. Images were recorded digitally with a Slow-Scan CCD-Camera (ProScan, 1024x1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany). Images were corrected for brightness and

contrast applying Adobe photoshop CS 3. This work was kindly done by Dr. Manfred Rohde and Ina Schleicher at the HZI, Braunschweig.

| | | |
|---|---|---|
| cacodylate buffer, pH 6.9 | cacodylate | 100 mM |
| | $CaCl_2$ | 10 mM |
| | $MgCl_2$ | 10 mM |
| | sucrose | 90 mM |
| 1x PBS, pH 7.4 | NaCl | 137 mM |
| | KCl | 2.7 mM |
| | $Na_2HPO_4$ x 7 $H_2O$ | 10 mM |
| | $KH_2PO_4$ | 2 mM |

## 2.5 Molecular Biological Techniques

### 2.5.1 Preparation of Plasmid DNA (Miniprep)

Cells from a 2 ml overnight culture were harvested by centrifugation (11'000 * *g*; 5 min). The sedimented cells were suspended in 150 µl of GTE solution and incubated for 5 min at room temperature (RT). For cell lysis, 300 µl of NaOH/SDS were added. The sample was carefully mixed by inverting the tube and incubated at RT for 2 min. 225 µl of NaAc were added and the sample was carefully mixed again. After centrifugation (11'000 * *g*; 25 min) the supernatant was mixed with 600 µl of isopropanol in a fresh tube and centrifuged (11'000 * *g*; 10 min). The sedimented DNA was suspended in 200 µl of TES solution containing 3 µl of RNase A and incubated for 1 h at 37 °C. Following an extraction with 200 µl CPI and vigorous shaking, the DNA was centrifuged (11'000 * *g*; 3 min). The DNA in the upper phase was precipitated again by addition of 400 µl of 100 % ethanol and centrifugation (11'000 * *g*; 20 min). Finally, the DNA precipitate was dried and solubilized in 50 µl $dH_2O$.

| | | |
|---|---|---|
| GTE solution | Tris-HCl, pH 8.0 | 25 mM |
| | ethylenediaminetetraacetic acid (EDTA) | 10 mM |
| | glucose | 50 mM |
| NaOH/SDS | NaOH | 200 mM |
| | SDS | 1 % (w/v) |
| NaAc | sodium acetate, pH 4.8 | 3 M |
| TES solution | Tris-HCl, pH 8.0 | 50 mM |
| | EDTA | 10 mM |
| | NaCl | 150 mM |

| | | |
|---|---|---|
| CPI | phenol | 50 % (v/v) |
| | chloroform | 49 % (v/v) |
| | isoamyl alcohol | 1 % (v/v) |
| RNase A | Ribonuclease A in 50 % (w/v) glycerol | 10 g/l |

### 2.5.2 Determination of DNA Concentration

The concentration and purity of a DNA solution was determined by measuring the absorbance at 260 nm and additionally at 280 nm to account for protein impurities. For a pure DNA solution, an $A_{260nm}$ of 1 corresponds to a concentration of 50 µg/ml dsDNA. The quality of the DNA solution is deduced from the ratio of $A_{260nm}$ to $A_{280nm}$. With $A_{260nm}/A_{280nm}$ = 1.8 - 2.0, the DNA can be considered as pure.

### 2.5.3 Agarose Gel Electrophoresis

For the analytical separation of DNA-fragments agarose gels (1 % (w/v) agarose in TAE-buffer) were prepared. DNA samples were mixed with DNA loading dye to facilitate loading and to indicate the progress of the samples in the gel. GeneRuler™ DNA Ladder Mix (MBI Fermentas, St. Leon-Roth, Germany) was used as DNA standard according to the manufacturer's instructions. Depending on the size of the gel, a voltage of 80 - 100 V was applied. The DNA fragments migrate towards the anode with a velocity that is proportional to the negative logarithm of their length. After electrophoresis, gels were incubated in an ethidium bromide solution for 30 min. The DNA was detected *via* its fluorescence under UV light (λ = 312 nm).

| | | |
|---|---|---|
| TAE buffer, pH 8.0 | Tris-acetate | 40 mM |
| | EDTA | 1 mM |
| DNA loading dye | bromophenol blue | 350 µM |
| | xylene cyanol FF | 450 µM |
| | glycerol | 50 % (w/v) |
| ethidium bromide solution | ethidium bromide | 0.1 % (w/v) |

### 2.5.4 Cloning of DNA

For the characterization of A-PGS$_{1\text{-}855N}$ and A-PGS$_{543\text{-}855N}$ proteins plasmids pBAD-His-A/PA0920 (Klein, 2007) and pGEX-6P-1/PA0920ΔAS1-542 (Tiefenau, 2007) were mutagenized by site-directed mutagenesis.
Furthermore, the alanyl-tRNA synthetase (AlaRS) encoding ORF PA0903 from *P. aeruginosa* was cloned into the pET28b(+) vector (Novagen, Darmstadt, Germany) to facilitate recombinant protein production in *E. coli*. The two isoacceptor tRNA$^{Ala}$ genes (PA4280.3, PA3133.2) from *P. aeruginosa* were cloned into pUC18 or pUC119 vectors that allowed for *in vitro* T7 RNA polymerase run-off transcription after plasmid cleavage with *Bst*NI (Sampson and Uhlenbeck, 1988). To study the overproduction of A-PGS in *P. aeruginosa*, a C-terminal His$_6$-tagged A-PGS construct was cloned into the *E. coli-P. aeruginosa* shuttle vector pUCP20T (West *et al.*, 1994). Furthermore, a mini-CTX2 based vector (Hoang *et al.*, 2000) for the chromosomal complementation of the *P. aeruginosa* deletion mutant ΔPA0920 was constructed.

#### 2.5.4.1 Amplification of DNA by Polymerase Chain Reaction (PCR)

For amplification of DNA by PCR, oligonucleotide primers were designed carrying appropriate recognition sequences for restriction endonucleases. Oligonucleotides used in this study are listed in Table 3.
PCR was performed in a total volume of 20 µl. After an initial DNA denaturation step (95 or 98 °C), a cycle consisting of denaturation, primer annealing (55 - 75 °C), and primer elongation (72 °C) was completed 30 times. PCR reactions were terminated by a final elongation period. The elongation step was adjusted according to the length of the DNA template and the employed DNA polymerase.

Standard thermocycler program for Phusion$^{TM}$ polymerase (Finnzymes, Espoo, Finland):

| | | |
|---|---|---|
| 5 min | 98 °C | |
| 30 sec | 98 °C | 30x |
| 30 sec | X °C | |
| 30 sec/kb | 72 °C | |
| 5 min | 72 °C | |

Standard thermocycler program for *Taq* DNA polymerase (Biotherm, Cologne, Germany):

| | | |
|---|---|---|
| 5 min | 95 °C | |
| 30 sec | 95 °C | 30x |
| 30 sec | X °C | |
| 60 sec/kb | 72 °C | |
| 5 min | 72 °C | |

#### 2.5.4.2 Restriction of DNA

Restriction of DNA (vectors and PCR products) was carried out using restriction endonucleases purchased from New England BioLabs (Ipswich, MA, USA). Reaction buffers, concentrations of enzymes, DNA concentrations as well as incubation temperatures were chosen according to manufacturer's instructions. The restriction proceeded for 1 - 3 h according to the employed enzymes.

#### 2.5.4.3 Purification of PCR Products and Vectors

After amplification of DNA by PCR, the resulting PCR products were purified using the "QIAquick PCR Purification Kit" (QIAgen, Hilden, Germany) according to the manufacturer's instructions.
Restriction enzymes were removed by agarose gel electrophoresis. DNA was visualized using the GelStar® Nucleic Acid Gel Stain (Biozym, Hessisch Oldendorf, Germany) on a blue light detector (Biozym, Hessisch Oldendorf, Germany) using a yellow filter. DNA fragments were then excised from the gel and purified using the "QIAquick Gel Extraction Kit" (QIAgen, Hilden, Germany) according to manufacturer's instructions.

#### 2.5.4.4 Ligation of DNA

Ligation of DNA was carried out using the T4 DNA ligase (MBI Fermentas, St. Leon-Roth, Germany) according to the manufacturer's instructions. An amount of 25 - 50 ng vector DNA was mixed with insert DNA (insert to vector ratio with regard to molar concentrations ≈ 5:1) in a final volume of 20 µl. Moreover, control reactions in the absence of insert or ligase were carried out. All reactions were incubated at RT for 2 h or at 17 °C overnight.

#### 2.5.4.5 Site-directed Mutagenesis (QuikChange)

Single amino acids or even multiple amino acid residues of a protein of interest can be exchanged by inserting site-specific mutations into the DNA sequence of the corresponding gene. The QuikChange® Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) was employed in this work for mutational analysis of truncated A-PGS variants (A-$PGS_{1\text{-}855N}$ and A-$PGS_{543\text{-}855N}$) from *P. aeruginosa*.
The method utilizes a dsDNA plasmid carrying the gene of interest and two synthetic oligonucleotide primers. The primers contain the desired mutation and are complementary to opposite strands of the vector. During PCR with the PfuTurbo DNA polymerase a mutated plasmid is generated. The PCR product is treated with *Dpn*I, an endonuclease specifically digesting partially methylated DNA, here the parental template DNA. This allows isolation of the newly *in vitro* synthesized unmethylated

DNA of the mutated plasmid with which a suitable *E. coli* strain is subsequently transformed. The DNA is then ligated by the DNA ligase of *E. coli*.
PCR reactions with the QuikChange® Site-directed Mutagenesis Kit were carried out according to the manufacturer's instructions. However, the volume of the PCR reaction was reduced to 25 µl. Competent *E. coli* DH10B cells were transformed with the mutated plasmids. Primers used for site-directed mutagenesis are listed in Table 3.

#### 2.5.4.6 Construction of Vectors

**Complementation of the ΔPA0920 deletion mutant**
For the complementation of the chromosomal ΔPA0920 deletion mutant (Lorenzo, 2006), a 2'947 bp PCR product covering 187 bp of the PA0920 upstream region, the PA0920 gene and 90 bp of PA0920 downstream region was amplified with the Phusion™ polymerase using primers 1PA0920SacIF and 2PA0920HindIIIR (primer annealing 75 °C). The PCR product was digested with *Sac*I and *Hin*dIII and ligated into vector mini-CTX2 (Hoang *et al.*, 2000) to generate mini-CTX2-PA0920.

**Construction of *P. aeruginosa* overexpression vectors for PA0920**
By using primers 3pET22b+BamHf and 4pET22b+KpnIr the PA0920 sequence flanked by the vector encoded T7 promoter region and a C-terminal $His_6$-tag region was amplified from plasmid pET22b(+)PA0920 (Piekarski, 2007) using the *Taq* DNA polymerase (annealing temperature 70 °C). The *Bam*HI/*Kpn*I-digested PCR fragment was cloned into the shuttle vector pUCP20T (West *et al.*, 1994) to yield plasmid pUCP20T-T7-PA0920-$His_6$.

**Construction of a *P. aeruginosa* alanyl-tRNA synthetase overexpression vector**
ORF PA0903 encoding the AlaRS from *P. aeruginosa* was amplified from genomic DNA using the Phusion™ polymerase with primers 27alaSNdeIfw and 28alaSBclIrv (annealing temperature 75 °C). The PCR fragment was digested with *Nde*I and *Bcl*I and cloned into the *Nde*I/*Bam*HI site of pET28b(+) to yield pET28b(+)alaRS.

**Construction of vectors for the *in vitro* transcription of $tRNA^{Ala}$ from *P. aeruginosa***
The two isoacceptor $tRNA^{Ala}$ genes (PA4280.3, PA3133.2) from *P. aeruginosa* were cloned using a hybridization method of oligonucleotides. Therefore, two complementary oligonucleotides were designed containing a 5' *Eco*RI site, the T7 promoter sequence, the respective $tRNA^{Ala}$ gene sequence, a 3' *Bst*NI and *Bam*HI site. One nmol of each oligonucleotide was phosphorylated using 20 units of T4 polynucleotide kinase (New England BioLabs, Ipswich, MA, USA) in T4 DNA Ligase Buffer (New England BioLabs, Ipswich, MA, USA) at 37 °C for 1 h. Stochiometric amounts of phosphorylated oligonucleotides alatRNA1fw and alatRNA1rv, or alatRNA2fw and alatRNA2rv were hybridized by incubation at 100 °C

for 5 min followed by a cooling step to 25 °C for 30 min. The resulting DNA fragments (containing sticky ends compatible of *Eco*RI and *Bam*HI restriction sites) were cloned into the *Eco*RI-*Bam*HI site of pUC18 or pUC119.

**Construction of vectors for production of truncated A-PGS variants from *P. aeruginosa***

Vectors pGEX-6P-1/A-PGS$_{543\text{-}855N}$ and pBAD-His-A/PA0920$_{1\text{-}855N}$ were constructed by site-directed mutagenesis with the QuikChange® Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instruction using pGEX-6P-1/PA0920ΔAS1-542 and pBAD-His-A/PA0920, respectively, as templates. The mutation of alanine residue 856 to asparagine and also the incorporation of a stop codon were facilitated by using primers QCP856NSTOPfw and QCP856NSTOPrv.

### 2.5.5 Preparation of Competent *E. coli* cells

#### 2.5.5.1 The $CaCl_2$ Method

An overnight culture of *E. coli* cells was inoculated in a ratio of 1 : 100 and grown in 100 ml LB medium under aerobic conditions. The bacteria were incubated at 37 °C and 200 rpm in baffled flasks until the culture reached an $OD_{578}$ of 0.6. Then, cells were harvested by centrifugation (2'900 * *g*; 10 min; 4 °C) and after washing with 10 ml of ice-cold $CaCl_2$ solution, cells were suspended in 1 ml $CaCl_2$ solution and stored at -80 °C.

| | | |
|---|---|---|
| $CaCl_2$ solution | $CaCl_2$ | 100 mM |
| | glycerol | 10 % (w/v) |

#### 2.5.5.2 The RbCl Method

*E. coli* cells were grown aerobically in 500 ml of LB medium containing the required additives. When the culture reached an $OD_{578}$ of 0.6 cells were harvested by centrifugation (2'900 * *g*; 10 min; 4 °C), suspended in 100 ml TFB1 and incubated on ice for 5 min. After subsequent centrifugation (2'900 * *g*; 10 min; 4 °C) cells were suspended in 2 volumes of TFB2 (referring to the volume of the cell sediment) and incubated on ice for 60 min. These cells were either immediately used for transformation or stored at -80 °C.

| | | |
|---|---|---|
| TFB1 | potassium acetate | 30 mM |
| | $CaCl_2$ | 10 mM |
| | $MnCl_2$ | 50 mM |
| | RbCl | 100 mM |
| | glycerol | 15 % (v/v) |
| | pH 5.8, adjusted with acetic acid | |
| TFB2 | piperazine-N,N'-bis(2-ethane sulfonic acid)-KOH, pH 6.5 | 10 mM |
| | $CaCl_2$ | 75 mM |
| | RbCl | 10 mM |
| | glycerol | 15 % (v/v) |

### 2.5.6 Transformation of Competent *E. coli* cells

The $CaCl_2$ and RbCl competent *E. coli* cells were transformed by heat shock. For this purpose, 1 – 10 µl of plasmid DNA (50 µg/ml) was mixed with 100 µl of competent *E. coli* cells and incubated on ice for 10 min. After heating the cells for 45 sec to 42 °C, cells were incubated for 2 min on ice. To regenerate the transformed cells, they were incubated in 500 µl of LB medium at 37 °C for 1 h. The transformation volume was plated on LB medium agar plates with appropriate antibiotics and incubated overnight at 37 °C.

### 2.5.7 Diparental Mating with *P. aeruginosa*

Transfer of mini-CTX derived plasmids into *P. aeruginosa* cells was carried out by diparental mating using *E. coli* ST18 (Thoma and Schobert, 2009) as donor. The CTX integrase of mini-CTX promoted the integration of the vector into the *attB* site of the *P. aeruginosa* genome. In the resulting strain parts of the mini-CTX vector containing the tetracycline resistance cassette were deleted using a FLP recombinase encoded on the pFLP2 plasmid (Hoang *et al.*, 1998) to yield markerless *P. aeruginosa* strains.
$CaCl_2$ competent *E. coli* ST18 cells were transformed with an *E. coli-P. aeruginosa* shuttle vector. One ml of an overnight culture of *E. coli* ST18 carrying the corresponding plasmid was sedimented (11'000 * *g*; 2 min; RT), suspended in 50 µl of an overnight culture of *P. aeruginosa*, and cultivated on a LB agar plate for 6 h at 37 °C. Bacteria were suspended in 400 µl of LB medium and selected on LB agar plates containing tetracycline. Subsequently, the tetracycline cassette was discarded from the *P. aeruginosa* genome. Therefore, in a second diparental mating the plasmid pFLP2 using *E. coli* ST18 was transferred into *P. aeruginosa.* Positive clones were selected by using carbenicillin LB agar plates. The pFLP2 encoded Flp recombinase enables the excision of the mini-CTX derived DNA sequences located in between two FRT

(Flippase Recognition Target) sites. To remove plasmid pFLP2 from *P. aeruginosa* cells were plated on LB agar plates containing 5 % (w/v) sucrose. Due to toxic accumulation of levan by the pFLP2 encoded levansucrase (*sacB*), cells without pFLP2 are selected. Colony PCR was performed to verify the correct insertion into the *P. aeruginosa* genome.

### 2.5.8 Sequencing

The successful modification of DNA (cloning as well as site-directed mutagenesis) was confirmed by sequence determination of the respective DNA region based on the Sanger method (Sanger *et al.*, 1977). The sequencing reactions were conducted with an ABI PRISM$^{TM}$ 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). The required PCR in the presence of fluorescence-labeled ddNTPs and the purification of the PCR product were carried out as described by the manufacturer. The analysis of all sequencing results was done using DNASTAR software package (GATC Biotech, Konstanz, Germany).

## 2.6 Protein Biochemical Methods

### 2.6.1 Recombinant Protein Production

#### 2.6.1.1 Production of A-PGS from *P. aeruginosa*

For recombinant production of the membrane protein A-PGS from *P. aeruginosa* in *E. coli*, 500 ml LB medium supplemented with ampicillin were inoculated (ratio 1:100) with an overnight culture of *E. coli* TOP10 carrying pBAD*myc*-His-A/PA0920 or pBAD-His-A/PA0920, respectively. The pBAD/His and pBAD/*myc*-His derived expression constructs allow for the tight regulated, dose-dependent recombinant protein production and purification. The employed *araBAD* promoter ($P_{araBAD}$) from *E. coli* allows for the tuning of protein production levels to obtain the recombinant peptide as a soluble protein. By varying the concentration of L-arabinose, protein production levels can be optimized. In addition, the tight regulation of $P_{araBAD}$ by AraC is important for the expression of potentially toxic genes (Guzman *et al.*, 1992).
Cultures were grown aerobically in LB medium supplemented with ampicillin at 37 °C and 180 rpm to an $OD_{578}$ of 0.5. Recombinant production of A-PGS was induced by the addition of L-arabinose to a final concentration of 0.02 % (w/v) for production of $His_6$-A-PGS or 0.75 % (w/v) for production of A-PGS-myc-$His_6$, respectively. Cells were cultivated for 4 h and subsequently harvested by centrifugation (2'900 * *g*; 10 min; 4 °C). Cell sediments were stored at -20 °C.

#### 2.6.1.2 Recombinant Production of Truncated A-PGS, L-PGS and Mutant Proteins

The truncated *P. aeruginosa* A-PGS variant A-PGS$_{543-881}$ and the corresponding mutant proteins (containing single amino acid exchanges), were produced as soluble GST-fusion proteins in *E. coli* BL21 (λDE3). Cultures were grown aerobically in LB medium supplemented with ampicillin at 37 °C and 180 rpm to an $OD_{578}$ of 0.5. Recombinant production of truncated variants and mutant proteins was induced by the addition of 50 µM IPTG. Protein production was performed at 37 °C for 3 h. Cells were harvested by centrifugation (2'900 * *g*; 10 min; 4 °C) and stored at -20 °C.
Analogously, truncated versions of L-PGS from *S. aureus* (MprF$_{515-840}$), *L. innocua* (Lin1803$_{512-865}$) and *L. monocytogenes* (Lmo1695$_{512-865}$) and *P. aeruginosa* A-PGS$_{543-855N}$ were produced by cultivation for 5 h at 17 °C after IPTG induction (50 µM).

#### 2.6.1.3 Production of lysyl-tRNA Synthetase from *B. burgdorferi* and alanyl-tRNA Synthetase from *P. aeruginosa*

For the establishment of an *in vitro* aa-PGS activity assay it was mandatory to produce und purify the corresponding aminoacyl-tRNA synthetases. Therefore, the lysyl-tRNA synthetase (LysRS) from *B. burgdorferi* was produced in *E. coli* Rosetta 2 (DE3) cells carrying vector pET15bLysS (Ibba *et al.*, 1997). The AlaRS from *P. aeruginosa* was produced in *E. coli* Rosetta (DE3) pLysS cells carrying plasmid pET28b(+)alaRS. Cultures were grown aerobically at 37 °C and 180 rpm to an $OD_{578}$ of 0.5. Recombinant production of lysyl-tRNA synthetase or alanyl-tRNA synthetase was induced by the addition of 200 µM IPTG after a temperature shift to 30 °C. After 5 h, cells were harvested by centrifugation (2'900 * *g;* 10 min; 4 °C). Cell sediments were stored at -20 °C.

#### 2.6.1.4 Production of histidyl-tRNA Synthetase and alanyl-tRNA Synthetase from *E. coli*

$His_6$-tagged *E. coli* AlaRS and HisRS were overproduced using the *E. coli* ASKA library clones JW2667 and JW2498 (Kitagawa *et al.*, 2005), respectively. Cultures were grown in LB medium with self inducing reagents (Studier, 2005) supplemented with chloramphenicol at 37 °C overnight. Cells were harvested by centrifugation (2'900 * *g;* 10 min; 4 °C).

#### 2.6.1.5 Production of A-PGS in *P. aeruginosa* ADD1976

ADD1976 (Brunschwig and Darzins, 1992) is a *P. aeruginosa* PAO1 derivative, which carries a chromosomal integrated T7 RNA polymerase gene under control of an inducible *lac*UV5 promoter. *P. aeruginosa* ADD1976 harboring pUCP20T-T7-PA0920-

$His_6$ for production of A-PGS-$His_6$ was cultivated at 37 °C in 500 ml of LB medium supplemented with carbenicillin to an $OD_{578}$ of 0.8. Protein production was induced by addition of 2 mM IPTG. Cells were further incubated for 4 h and harvested by centrifugation (2'900 * *g;* 10 min; 4 °C). Cell sediments were stored at -20 °C.

### 2.6.2 Disruption of Cells

For characterization of the A-PGS enzyme overproduced in *E. coli* or in *P. aeruginosa*, sedimented cells were suspended in lysis buffer 1 and disrupted by a double passage through a French® Press (Thermo Fisher Scientific, Waltham, Ma, USA) at 19'200 p.s.i..

| | | |
|---|---|---|
| lysis buffer 1 | Hepes-NaOH, pH 7.8 | 50 mM |

For analysis of the truncated aa-PGS variants sedimented cells were suspended in lysis buffer 2 or optional in lysis buffer 2 without 1,4-dithio-D,L-threitol (DTT) (chemical modification experiments) and disrupted by French® Press.

| | | |
|---|---|---|
| lysis buffer 2 | DTT | 2 mM |
| | in 1x PBS, pH 7.4 | |
| 1x PBS, pH 7.4 | NaCl | 137 mM |
| | KCl | 2.7 mM |
| | $Na_2HPO_4$ x 7 $H_2O$ | 10 mM |
| | $KH_2PO_4$ | 2 mM |

*E. coli* BL21 CodonPlus™ (DE3)-RIL overproducing T7 RNA polymerase were suspended in lysis buffer 3 and disrupted by French® Press.

| | | |
|---|---|---|
| lysis buffer 3 | Tris-HCl, pH 8.0 | 50 mM |
| | NaCl | 100 mM |
| | β-mercaptoethanol | 5 mM |
| | glycerol | 5 % (w/v) |

*E. coli* Rosetta (DE3) pLysS overproducing the AlaRS from *P. aeruginosa* and Rosetta 2 (DE3) pLysS overproducing the LysRS from *B. burgdorferi* were suspended in lysis buffer 4 and 5, respectively. Cells were disrupted by French® Press.

| | | |
|---|---|---|
| lysis buffer 4 | Tris-HCl, pH 7.2 | 50 mM |
| | NaCl | 300 mM |
| | $MgCl_2$ | 40 mM |
| | glycerol | 10 % (w/v) |
| lysis buffer 5 | Tris-HCl, pH 8.0 | 35 mM |
| | NaCl | 300 mM |
| | glycerol | 10 % (w/v) |

For purification of HisRS and AlaRS from *E. coli*, cells were suspended in lysis buffer 6 and broken by sonication (3x 1 min; 0.5 sec interval; amplitude 70 %; sonotrode MS73 (Bandelin, Berlin, Germany).

| | | |
|---|---|---|
| lysis buffer 6 | Tris-HCl, pH 7.0 | 50 mM |
| | NaCl | 200 mM |
| | $MgCl_2$ | 20 mM |
| | β-mercaptoethanol | 1 mM |

### 2.6.3 Isolation of Membrane Proteins

To determine the enzymatic activity of the full length A-PGS from *P. aeruginosa* it was mandatory to solubilize this membrane protein from the bacterial membrane. Therefore, a Triton X-100 based solubilization method was employed. Outer membrane proteins were separated from cytoplasmic membrane proteins by treatment with sarcosyl, a detergent which has been shown to selectively solubilize the cytoplasmic membrane proteins.

#### 2.6.3.1 Triton X-100 Solubilization

The full length A-PGS from *P. aeruginosa* was solubilized by Triton X-100. Therefore, after cell disruption the membrane fraction of *E. coli* TOP10 pBAD-His-A/PA0920 was sedimented by ultracentrifugation (110'000 * *g*; 1 h; 4 °C). To purify the membrane fraction from soluble proteins a discontinuous sucrose gradient centrifugation was performed. For this purpose, the membrane fraction was suspended in 2 ml of a 20 % (w/v) sucrose solution and was layered onto a three-step gradient consisting of a 60 %, 40 % and 20 % (w/v) sucrose solution (3 ml each). After centrifugation for 2 h at 145'000 * *g* and 4 °C in a swing-out-rotor (SW 40 Ti), the membrane fraction was enriched on the boundary layer of the 40 % (w/v) and 60 % (w/v) sucrose solution. The obtained membrane fraction was harvested and diluted in the same volume of

2x solubilization buffer. This suspension was stirred overnight and subsequently centrifuged (110'000 * *g;* 1 h; 4 °C). The supernatant contains solubilized membrane proteins.

| | | |
|---|---|---|
| sucrose solution | sucrose | 20/40/60 % (w/v) |
| | Tris-HCl, pH 8.0 | 10 mM |
| | $MgCl_2$ | 100 mM |
| 2x solubilization buffer | Tris-HCl, pH 8.0 | 100 mM |
| | $MgCl_2$ | 40 mM |
| | Triton X-100 | 18 M |

### 2.6.3.2 Sarcosyl Solubilization

Alternatively, the full length A-PGS from *P. aeruginosa* was solubilized by sarcosyl (sodium lauryl sarcosinate). Sarcosyl was found to disrupt selectively the cytoplasmic membrane of bacteria (Filip *et al.*, 1973). The preparation of the outer membrane fractions was performed according to Bölin *et al.* (1982). Therefore, L-PGS overproducing *E. coli* cells were suspended in 20 ml of TEM buffer and disrupted by French® Press. After ultracentrifugation (110'000 * *g*; 1 h; 4 °C) the sediment was suspended in 20 ml of SM buffer, stirred at 4 °C overnight and subsequently centrifuged (110'000 * *g*; 1 h; 4 °C). The evolving supernatant contained solubilized cytoplasmic membrane proteins. The outer membrane proteins containing sediment was suspended in 1x sample buffer.

| | | |
|---|---|---|
| TEM buffer | Tris-HCl, pH 8.0 | 10 mM |
| | EDTA | 5 mM |
| | β-mercaptoethanol | 1 mM |
| SM buffer | sarcosyl | 0.5 % (w/v) |
| | β-mercaptoethanol | 1 mM |
| 4x sample buffer | Tris-HCl, pH 6.8 | 62.5 mM |
| | SDS | 1 % (w/v) |
| | β-mercaptoethanol | 0.5 % (v/v) |
| | glycerol | 10 % (v/v) |

### 2.6.4 Affinity Chromatography of Cytosolic and Solubilized Membrane Proteins

For the affinity chromatography of cytosolic proteins, the cellular extract after cell disruption was centrifuged at 110'000 * *g* and 4 °C for 1 h. The resulting supernatant was loaded onto a gravity-flow column equilibrated with the appropriate lysis buffer.
For affinity chromatography of membrane proteins, the supernatant of the protein solubilization procedure was used.

#### 2.6.4.1 Affinity Chromatography of GST-tagged Proteins

For purification of GST-tagged proteins, a Poly-Prep chromatography column (BioRad, Munich, Germany) containing 1 ml of glutathione-Sepharose 4FF (GE Healthcare, Munich, Germany) was used. After applying the protein sample, the column was washed with 10 column volumes ($C_v$) of lysis buffer 2 (compare chapter 2.6.2) and 10 $C_v$ of Tris buffer. Alternatively, the column was washed with 20 $C_v$ of 1x PBS (2.4.5). The resin was incubated for 30 min with elution buffer A containing 10 mM glutathione, subsequently proteins were eluted using 4x 1 ml elution buffer A.

| | | |
|---|---|---|
| Tris buffer | Tris-HCl, pH 8.0 | 50 mM |
| | DTT | 2 mM |
| elution buffer A | glutathione<br>in Tris buffer or 1x PBS | 10 mM |

#### 2.6.4.2 Affinity Chromatography of $His_6$-tagged Proteins

For the purification of $His_6$-tagged proteins Chelating Sepharose FF (GE Healthcare, Munich, Germany) was used. The material was first loaded with 2 $C_v$ of 100 mM of $NiSO_4$ and washed with 5 $C_v$ of $H_2O$. The cytosolic extract or Triton X-100-solubilized membrane fractions after ultracentrifugation were applied onto nickel-loaded Chelating Sepharose FF. After extensive washing (10 $C_v$ of washing buffer) and an optional pre-elution step (lysis buffer containing 5 mM of imidazole), proteins were eluted in lysis buffer containing 500 mM or alternatively 1 M of imidazole.

### Purification of $His_6$-tagged Alanyl-tRNA Synthetase from *P. aeruginosa*

| | | |
|---|---|---|
| washing buffer B | Tris-HCl, pH 7.2 | 50 mM |
| | NaCl | 300 mM |
| | $MgCl_2$ | 40 mM |
| | glycerol | 10 % (w/v) |
| pre-elution buffer B | imidazole<br>in washing buffer B | 5 mM |
| elution buffer B | imidazole<br>in washing buffer B | 500 mM |

### Purification of $His_6$-tagged Alanyl-tRNA Synthetase and Histidyl-tRNA Synthetase from *E. coli*

| | | |
|---|---|---|
| washing buffer C | Tris-HCl, pH 7.0 | 50 mM |
| | NaCl | 200 mM |
| | $MgCl_2$ | 20 mM |
| | β-mercaptoethanol | 1 mM |
| elution buffer C | imidazole<br>in washing buffer C | 1 M |

### Purification of $His_6$-tagged T7 RNA polymerase

| | | |
|---|---|---|
| washing buffer E | Tris-HCl, pH 8.0 | 50 mM |
| | NaCl | 100 mM |
| | β-mercaptoethanol | 5 mM |
| | glycerol | 5 % (w/v) |
| pre-elution buffer E | imidazole<br>in washing buffer E | 5 mM |
| elution buffer E | imidazole<br>in washing buffer E | 500 mM |

### Purification of $His_6$-tagged A-PGS from Membrane Solubilized Fractions

| | | |
|---|---|---|
| washing buffer F | 2x solubilization buffer (2.6.3.1) | 50 % (v/v) |
| | imidazole | 5 mM |
| elution buffer F | 2x solubilization buffer | 50 % (v/v) |
| | imidazole | 500 mM |

### 2.6.5 Dialysis

Buffer exchange was performed at 4 °C using dialysis membranes with a molecular weight cut off of 14'000. Elution fractions were dialyzed three times against 500 - 1000 ml of the respective buffer without imidazole (14 h, 2 h, 2 h). T7 RNA polymerase containing elution fractions were dialyzed by dialysis membrane against storage buffer and stored at -80 °C.

| | | |
|---|---|---|
| storage buffer | Hepes-KOH, pH 8.0 | 20 mM |
| | NaCl | 100 mM |
| | EDTA | 1 mM |
| | DTT | 3 mM |
| | glycerol | 50 % (w/v) |

### 2.6.6 Protease Digestion for Removal of GST-Tag

GST-fusion tags of the purified recombinant proteins were proteolytically cleaved using the PreScission Protease site. Therefore, proteins bound to the resin were incubated overnight with the corresponding protease (10 units $mg^{-1}$) in 2 ml Tris buffer (chapter 2.6.4.1) or 1x PBS (chapter 2.4.5) at 4 °C.

## 2.7 Protein Characterization

### 2.7.1 Determination of Protein Concentration

Concentrations of purified proteins were determined using the Bradford reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. The assay is based on the colorimetric method developed by Bradford (1976). Bovine serum albumin was used as a standard.

### 2.7.2 Discontinuous SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were analyzed by SDS-PAGE as described by Laemmli (1970) with modifications by Righetti *et al.* (1990) for discontinuous SDS-PAGE.
Protein samples containing soluble proteins were supplemented with 2x SDS loading dye and incubated at 95 °C for 10 min. For the analysis of membrane proteins, samples were incubated at 40 °C for 30 min in 2x SDS loading dye. This variation was mandatory to avoid irreversible aggregation of membrane proteins.
Samples were loaded onto gels which were run at 45 mA until the band of bromophenol blue dye reached the lower end of the gel. During electrophoresis, proteins were first

focussed in the stacking gel and subsequently separated according to their relative molecular mass in the running gel. The size standard employed was the Protein Molecular Weight Marker (MBI Fermentas, St. Leon-Roth, Germany). For immunochemical detection of proteins, the protein standard PageRuler$^{TM}$ Prestained Protein Ladder (MBI Fermentas, St. Leon-Roth, Germany) was used. Subsequently, gels were stained with Coomassie Brilliant Blue G-250 and destained until distinct protein bands were visible. For documentation, gels were scanned and then dried between two cellophane foils for storage.

| | | |
|---|---|---|
| acrylamide stock solution (Roth) | acrylamide | 30 % (w/v) |
| | N,N'-methylen bisacrylamide | 0.8 % (w/v) |
| running gel, 12 % (w/v) | acrylamide stock solution | 2 ml |
| | 1.5 M Tris-HCl, pH 8.8 with 0.4 % (w/v) SDS | 1.25 ml |
| | $dH_2O$ | 1.75 ml |
| | 10 % (w/v) ammonium peroxodisulphate (APS) | 50 µl |
| | N,N,N',N',-tetramethyl ethylen diamine (TEMED) | 5 µl |
| running gel, 9 % (w/v) | acrylamide stock solution | 1.5 ml |
| | 1.5 M Tris-HCl, pH 8.8 with 0.4 % (w/v) SDS | 1.25 ml |
| | $dH_2O$ | 2.25 ml |
| | 10 % (w/v) APS | 50 µl |
| | TEMED | 5 µl |
| stacking gel, 6 % (w/v) | acrylamide stock solution | 0.5 ml |
| | 0.5 M Tris-HCl, pH 6.8 with 0.4 % (w/v) SDS | 625 µl |
| | $dH_2O$ | 1.375 ml |
| | 10 % (w/v) APS | 25 µl |
| | TEMED | 2.5 µl |
| electrophoresis buffer | Tris-HCl, pH 8.4 | 50 mM |
| | glycine | 380 mM |
| | SDS | 0.1 % (w/v) |
| 2x SDS loading dye | Tris-HCl, pH 6.8 | 50 mM |
| | glycerol | 10 % (v/v) |
| | β-mercaptoethanol | 2 mM |
| | SDS | 2 % (w/v) |
| | bromophenol blue | 0.1 % (w/v) |
| staining solution | acetic acid | 10 % (v/v) |
| | ethanol | 30 % (v/v) |
| | Coomassie Brilliant Blue | 0.25 % (w/v) |

| | | |
|---|---|---|
| destaining solution | acetic acid | 10 % (v/v) |
| | ethanol | 30 % (v/v) |

### 2.7.3 Immunochemical Detection of Proteins by Western Blot

For the immunochemical detection, electrophoretically separated proteins were transferred onto a polyvinylidene difluoride (PVDF - pore width 0.45 µm) membrane using a Trans Blot apparatus (semi dry transfer cell; BioRad, Munich, Germany). Therefore, the PVDF-membrane was activated in methanol for 15 min and then equilibrated in Towbin buffer. After SDS-PAGE, the unstained gel and two pieces of Whatman paper were equilibrated in Towbin buffer for 15 min. The proteins were blotted onto the membrane for 1 h at 10 V per gel using the semi dry method according to the manufacturer's instructions.
For detection of $His_6$-tagged or myc epitope containing A-PGS proteins, the membrane was blocked overnight in blocking buffer, and incubated for 1 h with murine anti-His antibody (GE Healthcare, Munich, Germany) or with murine anti-*myc*-antibody (Invitrogen, Darmstadt, Germany) which was previously diluted 1:5'000. After washing three times for 10 min in wash buffer, the membrane was incubated with the secondary anti-mouse antibody (1:10'000) conjugated with horse-radish peroxidase (Pierce, Bonn, Germany) for 45 min. Subsequently, the membrane was washed four times in PBS-Tween for 10 min and the chemiluminescence reaction was performed using the SuperSignal® West Pico Chemiluminescence Kit (Pierce, Bonn, Germany) according to manufacturer's instruction.

| | | |
|---|---|---|
| Towbin buffer | Tris-HCl, pH 9.5 | 25 mM |
| | glycine | 192 mM |
| PBS-Tween | Tween 20<br>in 1x PBS (2.4.5) | 0.1 % (v/v) |
| blocking buffer | skim milk powder<br>in PBS-Tween | 5 % (w/v) |
| wash buffer | skim milk powder<br>in PBS-Tween | 0.5 % (w/v) |

### 2.7.4 Determination of Native Molecular Mass

Analytical gel permeation chromatography was performed using a Superdex 75 HR 10/30 column (GE Healthcare, Munich, Germany) equilibrated with Tris buffer (chapter 2.6.4.1) supplemented with 250 mM NaCl. This column was calibrated using the

following marker proteins: carbonic anhydrase ($M_r$ ~ 29'000) and bovine serum albumin ($M_r$ ~ 66'000) (Molecular weight marker Kit, Sigma-Aldrich, St Louis, MO, USA). Then, 250 µl of the purified protein (3 - 5 mg $ml^{-1}$) was run under identical conditions at a flow rate of 0.5 ml $min^{-1}$. Protein elution was monitored by measuring the absorbance at 280 nm.

### 2.7.5 UV/Visible Light Absorption and Fluorescence Spectroscopy

For detection of possible cofactors of A-PGS UV-visible light spectra of purified recombinant A-$PGS_{543-881}$ were recorded from 260 - 900 nm using a V-550 spectrometer (Jasco, Groß Umstadt, Germany). Fluorescence spectra using the LS50B-luminescence spectrometer (Perkin Elmer, Boston, MA, USA) were monitored to detect possible fluorescent cofactors. Therefore, purified A-$PGS_{543-881}$ was excited from 250 - 450 nm. Fluorescence emission maxima were detected from 250 - 800 nm.

### 2.7.6 Inductively Coupled Plasma - Mass Spectrometry (ICP-MS)

To determine protein-bound metal ions ($Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Zn^{2+}$) commercial inductively coupled plasma - mass spectrometry (ICP-MS) was performed (CURRENTA Bayer-Analytics, Leverkusen, Germany). For this purpose, the affinity purification of GST-A-$PGS_{543-881}$ was performed in the absence of DTT.

### 2.7.7 Testing of promoter–*lacZ* Reporter Gene Fusion

For determination of β-galactosidase activities *P. aeruginosa* strains PAO1-$P_{PA0920}$-*lacZ* (Spier, 2007), PAO-SK03 and PAO-SK04 and control strains KS11 (Schreiber *et al.*, 2006), PAO-SK01 and PAO-SK02 were cultivated in pH-adjusted AB minimal media or additional in LB media over a time period of 24 h. Aerobic growth conditions were achieved by vigorous shaking (200 rpm) of 60 ml cultures in 300 ml baffled flasks at 37 °C. Samples at different time points were centrifuged (11'000 * *g*; 5 min; RT) and suspended in 800 µl of Z buffer. Cells were disrupted by adding one drop of 0.1 % (w/v) SDS and one drop of chloroform, respectively, and incubation for 5 min at 30 °C. The β-galactosidase assay was initiated by addition of 200 µl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg $ml^{-1}$) at 30 °C. The reaction was stopped using 500 µl of 1 M $Na_2CO_3$ when a yellow coloring of the assay was observed. The required time for this enzymatic reaction was determined. After centrifugation (11'000 * *g*; 5 min; RT), the supernatant was photometrically analyzed at 420 nm.

| | | |
|---|---|---|
| Z buffer | $Na_2HPO_4$ | 60 mM |
| | $NaH_2PO_4$ | 40 mM |
| | KCl | 10 mM |
| | $MgSO_4$ | 1 mM |
| | β-mercaptoethanol | 50 mM |

β-galactosidase activity was expressed in Miller units (MU) (Miller, 1992) using the following equation:

$$\text{activity in MU} = \frac{\Delta E_{420} \cdot 1000}{OD_{578} \cdot V \cdot t}$$

| | |
|---|---|
| $E_{420}$: | extinction at a wavelength of 420 nm |
| $OD_{578}$: | optical density of the employed cell culture |
| V [ml]: | volume of the employed cell culture |
| t [min]: | time of enzymatic reaction |

Obtained values of β-galactosidase activity are the averages of at least three independent experiments.

## 2.8 Lipid Analysis

### 2.8.1 Extraction and Analysis of Polar Membrane Lipids by two-dimensional Thin Layer Chromatography and Liquid Scintillation Counting

Whole bacterial cells or alternatively membrane fractions from an A-PGS activity assay were harvested by centrifugation for 5 and 30 min at 11'000 * *g*, respectively. The sediment was used for a modified extraction method of polar membrane lipids according to Bligh and Dyer (1959). Hence, the sediment was suspended in 100 µl of $dH_2O$, 375 µl of methanol/chloroform (2:1, by volume) was added, mixed vigorously and incubated for 5 min at RT. After centrifugation (11'000 * *g*; 3 min; RT) the supernatant was mixed with 200 µl of chloroform/$dH_2O$ (1:1, by volume), centrifuged (11'000 * *g*; 3 min; RT) and the lower chloroform phase was harvested and dried at 40 °C. The obtained lipids were suspended in methanol/chloroform (1:1, by volume) and subjected to two-dimensional thin layer chromatography (2D-TLC) or used for scintillation analysis.
For 2D-TLC, silica thin-layer plates "DC-plates Alugram® SIL G/UV254" (Macherey-Nagel, Düren, Germany) were loaded with appropriate amounts of lipid extracts and run in the first dimension with chloroform/methanol/water (60:25:4, by volume), and in the second dimension with chloroform/methanol/acetic acid/water (80:12:15:4, by volume) (Tindall, 1990). Total polar lipids were detected by spraying with 5 % (w/v) molybdatophosphoric acid (Merck, Darmstadt, Germany) and subsequently by carbonization at 120 °C for 5 min. Free amino groups were detected using 0.3 % (w/v)

ninhydrin solution (Merck, Darmstadt, Germany) and carbonization at 110 °C for 5 min. Phospholipids were identified by spraying the plate previously used for the detection of free amino groups with 1.3 % (w/v) Molybdenum Blue Spray reagent (Sigma-Aldrich, St. Louis, MO, USA).
Radioactively labeled phospholipids were analyzed by autoradiography of 2D-TLC or by scintillation counting. Therefore, the lipid fraction was added to 4 ml of OptiPhase HighSafe 2 (Perkin Elmer, Boston, MA, USA) and counts per minute were detected using a TriCarb 2900 TR scintillation counter (Perkin Elmer, Boston, MA, USA).

### 2.8.2 Biophysical Lipid Analyses

2D-TLC-isolated polar lipids were dissolved in methanol/chloroform/water (6:3:1, by volume) prior positive and negative ion electrospray mass spectrometric analysis (ESI-MS). A voltage of approximately 1'000 V was applied to the nanospray capillary, and ions were subjected to ESI-MS on a QTOF 2 mass spectrometer (Micromass, Manchester, UK). For tandem mass spectrometry (MS/MS) analysis, parent ions were selected by the quadrupole mass filter and subjected to collision-induced dissociation. The resulting daughter ions were separated by the TOF analyzer. A $^{1}H$ nuclear magnetic resonance (NMR) spectrum of the sample used for MS analysis was recorded on a Bruker AVANCEIII 600 NMR spectrometer (Bruker, Karlsruhe, Germany) with a cyroprobehead locked to the major deuterium signal of the $CD_3OD$ solvent. Chemical shifts were referenced to the residual $^{1}H$ signal of the solvent at 3.35 p.p.m.. Additionally, the residue linked to the second glycerol moiety was determined by gas chromatography coupled with mass spectrometry (GC/MS) analysis after hydrolysis and derivatization with isopropanol followed by pentafluoropropionic acid anhydride using a Chirasilval column (50 m) connected to a GCQ ion trap mass spectrometer (Thermo Electron Corporation, Erlangen, Germany). The fatty acid composition of the isolated lipid was confirmed by GC/MS of the fatty acid methyl esters. These were released after acid catalyzed methanolysis of the intact lipid. This work was kindly done by Dr. Manfred Nimtz and Prof. Dr. Victor Wray at the HZI, Braunschweig.

## 2.9 tRNA Methods

For A-PGS activity assays, the tRNA substrate was purchased (tRNA Mix from *E. coli*) or produced by *in vitro* transcription. These tRNAs were either subjected directly to coupled AlaRS/A-PGS activity assays or were aminoacylated with radioactively labeled amino acids and subsequently subjected to an activity assay.

### 2.9.1 *In vitro* Transcription and Purification of tRNA

For *in vitro* transcription of $tRNA^{Ala}$, template DNA was prepared by *Bst*NI digestion of 500 µg of the respective plasmid in NEB Buffer 2 (New England BioLabs, Ipswich, MA, USA), 1x BSA and with 150 units of *Bst*NI in a total volume of 250 µl. After incubation at 60 °C for 3 h, the reaction mixture was precipitated using 100 µl 3 M sodium acetate (pH 4.8) and 625 µl ethanol at -20 °C overnight. The precipitated plasmid DNA was used for *in vitro* transcription in a reaction volume of 250 µl containing 40 mM Hepes-KOH (pH 7.8), 22 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 3 mM of each ribonucleotide triphosphate (rNTP), and 200 nM purified T7 RNA polymerase at 37 °C overnight. Efficient *in vitro* transcription was clearly indicated by the visible precipitation of $PP_i$.
For purification of tRNA transcripts MonoQ anion exchange chromatography (MonoQ 5/50 GL (Pharmacia Biotech, Munich, Germany)) with a flow rate of 0.5 ml $min^{-1}$ and a 20 ml gradient (0 - 750 mM NaCl) of MonoQ-buffer A and B was used according to Jahn *et al.* (1991). Subsequently, 500 µl tRNA fractions were precipitated by 1.25 ml ethanol and 200 µl of 3 M sodium acetate (pH 5.2) and centrifugation (2'900 * *g*; 1 h; 4 °C). Alternatively, tRNA transcripts were purified *via* 12 % polyacrylamide gel electrophoresis in the presence of 8 M urea according to Pande *et al.* (1991). Therefore, tRNA *in vitro* transcription reactions were supplemented with 2x tRNA loading dye and loaded onto a polyacrylamide gel and allowed to run at 18 watt overnight. tRNA bands were excised after visualization by UV detection with a portable UV lamp. Gel extraction was performed by the addition of 1 M sodium acetate (pH 5.0) and shaking at 4 °C. Subsequently, the tRNA was precipitated by addition of one volume isopropanol to the liquid phase and centrifugation (6'000 * *g*; 1 h; 4 °C). Precipitated tRNA was dissolved in $dH_2O$.

| | | |
|---|---|---|
| MonoQ-buffer A | MOPS-KOH, pH 6.2 | 20 mM |
| MonoQ-buffer B | MOPS-KOH, pH 6.2 | 20 mM |
| | NaCl | 1.5 M |
| acrylamide stock solution (Roth) 40 % (w/v) | acrylamide | 38 % (w/v) |
| | N,N'-methylen bisacrylamide | 2 % (w/v) |
| polyacrylamide gel (12 %) | acrylamide stock solution 40 % (w/v) | 300 ml |
| | urea | 500 g |
| | 10x TBE buffer | 100 ml |
| | 10 % (w/v) APS | 3 ml |
| | TEMED | 300 µl |
| | | $dH_2O$ added to volume of 1000 ml |

| | | |
|---|---|---|
| 10x TBE buffer Tris | Tris | 1 M |
| | Boric acid | 850 mM |
| | EDTA | 100 mM |
| 2x tRNA loading dye | urea | 8 M |
| | bromophenol blue | 250 µg/ml |
| | xylene cyanol | 250 µg/ml |
| | sucrose | 20 % (w/v) |

### 2.9.2 Denaturating Polyacrylamide Gel Electrophoresis of tRNAs

tRNAs were analyzed by denaturating polyacrylamide gel electrophoresis. Therefore, samples were supplemented with 2x tRNA loading dye. The included urea inhibits the formation of secondary and tertiary structures. Samples were loaded onto 10 % (w/v) polyacrylamide gels which were run at 30 watt until the band of bromophenol blue dye dropped out of the lower end of the gel. As size standard the tRNA Mix from *E. coli* (Sigma-Aldrich, St. Louis, MO, USA) was employed. Subsequently, gels were stained with toluidine blue (tRNA staining solution) for 5 min and destained with water until distinct tRNA bands were visible. For documentation, gels were scanned and dried between two cellophane foils for storage.

| | | |
|---|---|---|
| polyacrylamide gel, 10 % (w/v) | acrylamide stock solution 40 % (w/v) | 2.5 ml |
| | urea | 4.8 g |
| | 10x TBE buffer | 1 ml |
| | 10 % (w/v) APS | 60 µl |
| | TEMED | 10 µl |
| | $dH_2O$ added to volume of 10 ml | |
| tRNA staining solution | acetic acid | 1 % (v/v) |
| | methanol | 40 % (v/v) |
| | toluidine blue | 2.5 mM |

### 2.9.3 Aminoacylation of tRNA and tRNA Microhelices

Purified, synthetic tRNA microhelices were purchased from Integrated DNA Technologies (Coralville, IA, USA). tRNA *in vitro* transcripts and microhelices were denatured at 80 °C for 2 min and subsequently incubated at 60 °C for 2 min, followed by addition of 10 mM $MgCl_2$ and immediate cooling on ice to facilitate RNA folding.
AlaRS aminoacylation reactions were performed at RT according to Swairjo *et al.* (2004) in aminoacylation buffer A using 4.75 µM of [2,3-$^{3}$H]-L-alanine (52 µCi µmol$^{-1}$, GE Healthcare, Munich, Germany) and 20 µM of [1-$^{14}$C]-L-alanine (51 µCi µmol$^{-1}$, Moravek Biochemicals, Brea, CA), respectively, 10 µM of tRNA [tRNA Mix from

*E. coli* strain W (Sigma-Aldrich, St. Louis, MO, USA) or *in vitro* transcribed $tRNA^{Ala}$] or microhelices and 1 µM of purified AlaRS. At different time points samples were spotted onto Whatman filters (Whatman, Munich, Germany) and the tRNA was precipitated by incubation of the filters in 5 % (w/v) trichloroacetic acid for 10 min. Subsequently filters were subjected to 4 ml scintillation liquid OptiPhase High Safe 2 (Perkin Elmer, Boston, MA, USA) and counts per minute were detected using a TriCarb 2900 TR scintillation counter (Perkin Elmer, Boston, MA, USA).

| | | |
|---|---|---|
| aminoacylation buffer A | Hepes-NaOH, pH 7.5 | 50 mM |
| | KCl | 20 mM |
| | $MgCl_2$ | 10 mM |
| | BSA | 0.1 mg/ml |
| | β-mercaptoethanol | 20 mM |
| | ATP | 4 mM |

HisRS aminoacylation reaction was carried out in aminoacylation buffer B at 37 °C according to Connolly *et al.* (2004) containing 21.7 µM of [U-$^{14}$C]-L-histidine (322 µCi µmol$^{-1}$, GE Healthcare, Munich, Germany), 10 µM of microhelix F and 1 µM of purified HisRS.

| | | |
|---|---|---|
| aminoacylation buffer B | Hepes-NaOH, pH 7.5 | 50 mM |
| | $MgCl_2$ | 10 mM |
| | BSA | 100 µg/ml |
| | DTT | 5 mM |
| | ATP | 2 mM |

### 2.9.4 Purification of Aminoacylated tRNAs and tRNA Microhelices

Aminoacylated tRNAs were purified by a phenol/chloroform extraction. To inhibit hydrolysis of the aminoacyl-bond acidic phenol (pH 4.5 - 5.0) was used. Furthermore, purification steps were performed as quickly as possible with cold solutions.
For purification of aminoacylated tRNAs, the aminoacylation reaction (1 ml) was supplemented with 4 ml 375 mM sodium acetate (pH 5.2), mixed and subsequently supplemented with 5 ml PCI solution, mixed again vigorously and centrifuged (11'000 * *g*; 1 min; 4 °C). To the upper phase 5 ml chloroform were added, mixed again and centrifuged. Aminoacylated tRNA was precipitated by addition of 15 ml ethanol to the upper phase and centrifugation (11'000 * *g*; 25 min; 4 °C). The tRNA sediment was dried by vacuum centrifugation, suspended in 100 µl of 30 mM sodium acetate (pH 4.9) and stored at -20 °C.

| | | |
|---|---|---|
| PCI solution | phenol | 25 ml |
| | chloroform | 24 ml |
| | isoamyl alcohol | 1 ml |

## 2.10 aa-PGS Activity Assays

For determination of activity of the recombinantly produced aa-PGS variants, *in vivo* and *in vitro* activity assays were established. The product of aa-PGS catalysis - aminoacylated PG - was isolated by lipid extraction and either detected by 2D-TLC or by liquid scintillation analysis. In all cases, assays were completed by control experiments in which the enzyme or the tRNA substrate was omitted. Alternatively, the assay mixture was pre-treated with 100 µg $ml^{-1}$ of RNase A.

### 2.10.1 Determination of *in vivo* aa-PGS Activity

For determination of the *in vivo* aa-PGS activity 5 ml of *E. coli* cells overproducing aa-PGS proteins or *P. aeruginosa* cells were harvested by centrifugation. Alternatively, cells were disrupted by French® Press and incubated with 2.8 mM - 44 mM of [U-$^{14}$C]-L-alanine or [1-$^{14}$C]-L-alanine (Moravek Biochemicals, Brea, CA, USA) and 2.8 mM [U-$^{14}$C]-L-lysine (Moravek Biochemicals, Brea, CA, USA), respectively, in the presence of an ATP-regenerating system (containing 2 mM of ATP, 18 mM of creatin phosphate and 35 U $ml^{-1}$ creatin phosphokinase) for 1 h at 37 °C and 1'000 rpm. Then lipids were extracted and separated by 2D-TLC. Radioactively labeled phospholipids were visualized by autoradiography.

### 2.10.2 Determination of *in vitro* aa-PGS Activity Using *E. coli* Extracts To Provide PG and aa-tRNA

For the *in vitro* activity assay using *E. coli* extracts an *E. coli* crude cellular extract was employed to provide the substrate molecules PG and aa-tRNA. Therefore, 500 µl of a crude cellular extract overproducing AlaRS or LysRS *E. coli* cells (compare chapter 2.6.1.4) were supplemented with 2 - 20 µM of purified GST-A-$PGS_{543-881}$ (or GST-A-$PGS_{543-855N}$, GST-Sa-L-$PGS_{515-840}$, GST-Lm-L-$PGS_{511-865}$, GST-Li-L-$PGS_{512-865}$), 2 mM of ATP, an ATP-regenerating system (consisting of 18 mM of creatine phosphate and 35 U $ml^{-1}$ of creatine phosphokinase) and 20 µM of [1-$^{14}$C]-L-alanine (Moravek Biochemicals, Brea, CA, USA) and [U-$^{14}$C]-L-lysine (Moravek Biochemicals, Brea, CA, USA), respectively, in a total volume of 700 µl at 37 °C under vigorous shaking (1'000 rpm). Samples of 100 µl volume were heat inactivated (5 min at 60 °C) and subjected to lipid extraction.
All assays for the determination of A-$PGS_{543-881}$ mutant activities were standardized by analyzing the individual proteins at a concentration of 10 µM for 1 h at 37 °C. The activities for wild type GST-A-$PGS_{543-881}$ obtained were set as 100 %. All other values of mutant GST-A-$PGS_{543-881}$ were related to this. Experiments were performed in triplicate and were completed by control experiments in which GST-A-$PGS_{543-881}$ was

omitted or in which the crude cellular extract was pre-treated with 100 µg $ml^{-1}$ of RNase A for 25 min.
To determine the specific activity of full length $His_6$-A-PGS the purified and solubilized protein was incubated with membrane fragments of a crude cellular extract of *E. coli* Rosetta (DE3) pLysS overproducing the AlaRS from *P. aeruginosa*.

### 2.10.3 Determination of *in vitro* A-PGS Activity

The *in vitro* A-PGS activity was determined by using purified aminoacylated tRNA or alternatively in a coupled AlaRS/A-PGS assay. For the *in vitro* A-PGS assay with aminoacylated tRNA, 0.2 - 5 µM of purified GST-A-$PGS_{543-881}$ and 2 - 20 nM of aminoacylated tRNA were added to a commercially available PG fraction [2 mg $ml^{-1}$ PG from egg yolk lecithin (Sigma-Aldrich, St Louis, MO, USA)] supplemented with 1.76 mg $ml^{-1}$ Triton X-100 in 50 mM Tris-HCl, pH 8.0. For the coupled AlaRS/A-PGS *in vitro* assay the crude cellular extract of the above mentioned *in vitro* activity assay using *E. coli* extracts was substituted with 5 µM of purified AlaRS, 10 - 15 µM of tRNA, and 2.7 mM of PG in aminoacylation buffer A (chapter 2.9.3) according to Swairjo *et al.* (2004). Samples of 100 µl volume were inactivated by the addition of 375 µl of MeOH/chloroform (2:1, v/v) and subsequently subjected to lipid extraction. Radioactively labeled phospholipids were detected by autoradiography.

### 2.10.4 Analysis of A-PGS Substrate Recognition by using Artificial PG Derivatives

Artificial lipids were employed to identify essential determinants of the PG substrate. For this purpose the coupled AlaRS/A-PGS *in vitro* assay was performed in the presence of PG derivatives (Avanti Polar Lipids, Inc., Alabaster, Al, USA) at a concentration of 2 mg $ml^{-1}$.

### 2.10.5 Analysis of A-PGS Substrate Recognition by using Artificial tRNA Substrates

To determine the tRNA identity elements of A-PGS catalysis *P. aeruginosa* wild type *in vitro* transcribed $tRNA^{Ala1}$ and tRNA microhelices were aminoacylated and subjected to an *in vitro* assay containing 5 µM of GST-A-$PGS_{543-881}$ in a total volume of 575 µl. The obtained A-PGS activity using *in vitro* transcribed $tRNA^{Ala1}$ was set as 100 %. Experiments were performed in triplicate. Standard deviations were ± 10 %. Microhelices which were inefficiently aminoacylated by AlaRS were tested as substrate

for A-PGS catalysis in a coupled AlaRS/A-PGS *in vitro* assay. Therefore, microhelices (20 µM) were pre-incubated at 37 °C for 30 min in the standard AlaRS aminoacylation buffer A (500 µl) (chapter 2.9.3). A-PGS catalysis was then initiated by the addition of 500 µl of aminoacylation buffer A containing 5.4 mM of PG (supplemented with 1.76 mg $ml^{-1}$ Triton X-100) and 10 µM GST-A-$PGS_{543-881}$. Experiments were performed in duplicate. This work was done in cooperation with Dr. Ilka Heinemann at the Yale University, New Haven.

### 2.10.6 Chemical Modification of A-$PGS_{543-881}$

To characterize potential amino acid residues with relevance for the A-PGS reaction mechanism, 10 pmol of A-$PGS_{543-881}$ were chemically modified with reagents showing a high degree of specificity for individual amino acids. For this purpose, the affinity purification of GST-A-$PGS_{543-881}$ was performed in the absence of DTT. After extensive washing of the column with 1x PBS (20 $C_v$) the target protein was liberated from the column by PreScission Protease treatment. The obtained eluate fraction (~ 60 µM) was incubated in the presence of phenylmethylsulfonyl fluoride (PMSF) (0.1, 1 mM), diisopropyl fluorophosphate (DIPFP) (0.1, 1 mM) and iodacetamide (1, 10 mM) at 4 °C for 45 min, or alternatively in the presence of citraconic anhydride (2, 10 mM) and p-hydroxyphenylglyoxal (HPG) (0.5, 1, 5, 10 mM) at 20 °C in the dark for 45 min. These protein fractions were excessively dialyzed against 1x PBS using Slide-A-Lyzer® MINI dialysis units (Pierce, Bonn, Germany) and subjected to *in vitro* activity assays using *E. coli* extracts in the presence of 10 mM ATP (ATP-regenerating system omitted) or to *in vitro* assays using aminoacylated $tRNA^{Ala}$. To study the influence of metal chelating agents the *in vitro* activity assays using *E. coli* extracts and *in vitro* assay was incubated in the presence of EDTA (3, 10, 20 mM) or 1,10-phenanthroline (1, 3, 5, 10, 20 mM), respectively.

## 2.11 Phenotypic Determination

The *P. aeruginosa* ΔPA0920 deletion mutant was tested for biofilm formation, motility or for the ability of excretion of extracellular proteins. Furthermore, a commercial available test system with over thousand culture conditions was employed.

### 2.11.1 Standard Phenotype Assays

Swimming and swarming motility were assayed on AB minimal medium agar plates containing 0.3 % and 0.5 % (w/v) agar according to O'Toole & Kolter (1998). The

plates were inoculated with a single colony using a toothpick. After incubation of the swimming plates for 24 h and swarming plates for 48 h at 37 °C the motility zone diameter was measured. Twitching motility was tested using thin LB agar plates, which were inoculated with a single colony by a toothpick down to the agar-plastic interface, and measuring the twitch zone diameter after 24 h of incubation at 37 °C and 48 h at RT as described (Köhler *et al.*, 2000). For static microtitre biofilms a 24 h LB overnight culture was diluted with fresh LB medium to yield $10^6$ cells per ml, and 200 µl was inoculated into wells of a 96-well polystyrene round-bottom microtitre plate (Nunc, Roskilde, Denmark). Plates were then incubated at 37 °C without shaking for 24 h. Medium and planktonic cells were discarded. Surface-attached bacteria were stained with 0.1 % (w/v) crystal violet for 15 min, followed by washing and ethanol solubilization of crystal violet-stained cells for quantification of $A_{600}$ (O'Toole and Kolter, 1998). The screening for extracellular enzymes was performed by spotting cells onto Difco™ DNase Test Agar with Methyl Green (Difco Laboratories, Detroit, MI) for DNase activity or onto agar plates containing 0.1 % (w/v) Elastin (Sigma-Aldrich, St. Louis, MO, USA) for elastase activity, 5 % (w/v) sheep red blood for haemolysin activity, 1 % (w/v) egg yolk (Fluka, Buchs, Switzerland) for phospholipase A and C activity and 5 % (w/v) skim milk powder (Roth, Karlsruhe, Germany) for protease activity, respectively, and incubation at 37 °C for 24 h.

### 2.11.2 Phenotyping of *P. aeruginosa* ΔPA0920 using Biolog Microarray

*P. aeruginosa* wild type and the corresponding ΔPA0920 deletion mutant strain were tested for phenotypic changes in MicroPlates™ PM9 – PM20 of the Phenotype Microarray system from Biolog using IF-10 medium (Biolog, Hayward, CA, USA) as described by Zhou *et al.* (2003) according to manufacturer's instruction. The employed test system is based on the reduction of a tetrazolium dye. Plates PM9 - PM20 measure sensitivities to salt and pH stresses and to a wide variety of antibiotics, antimetabolites and other inhibitors. The phenotypic changes were monitored visual by color change based on the reduction of a tetrazolium dye. Conditions showing significant alterations in the phenotypic behavior for the mutant strain were subjected to a second independent experiment. For this purpose, relevant conditions were reproduced. *P. aeruginosa* wild type, ΔPA0920 deletion mutant and the complemented ΔPA0920 deletion mutant (strain ΔPA0920compl) were mixed with $CrCl_3$ (Riedel-de-Haën, Selze, Germany) (3, 3.5 and 4 mM), cefsulodin (Sigma-Aldrich, St. Louis, MO, USA) (17.5, 20 and 25 mM), protamine sulphate (Roth, Karlsruhe, Germany) (4.2, 5.6 and 8.3 mM) and sodium lactate (Fluka, Buchs, Switzerland) (535, 625 and 714 mM) in a volume of 240 µl of IF-10 medium (Biolog, Hayward, CA, USA) containing tetrazolium dye and incubated in 96-well plates at 37 °C. At different time points, the $A_{600}$ was measured using a Fusion™ plate reader (Perkin Elmer, Boston, MA, USA).

# 3 RESULTS AND DISCUSSION

In this thesis the biological function of open reading frame (ORF) PA0920 from *Pseudomonas aeruginosa* and the biochemistry of its encoded protein were characterized. For this purpose, the transcriptional regulation of ORF PA0920 was investigated. This was followed by a phenotypic comparison of *P. aeruginosa* wild type and a ΔPA0920 deletion strain. The biochemical reaction mechanism of the encoded enzyme responsible for alanyl-phosphatidylglycerol synthesis was studied. Finally, the subcellular localization was determined.

## 3.1 Bioinformatical Analyses of Alanyl-Phosphatidylglycerol Synthase from *Pseudomonas aeruginosa*

Aminoacyl-phosphatidylglycerol synthases (aa-PGS) are enzymes catalyzing the modification of the phospholipid phosphatidylglycerol with an aminoacyl-moiety.

Basic Local Alignment Search Tool (BLAST) analysis (Altschul *et al.*, 1990) using the *Staphylococcus aureus* MprF amino acid sequence as query identified ORF PA0920 potenially encoding an aa-PGS protein in the genome of *P. aeruginosa*. After overproduction of the ORF PA0920 gene product in *E. coli* TOP10 a new amino group containing phospholipid was observed in the membrane of the *E. coli* host (Lorenzo, 2006; Klein, 2007). This lipid was identified in MS/MS analysis as alanyl-phosphatidylglycerol (A-PG) (Piekarski, 2007). So, the PA0920 protein was renamed to alanyl-phosphatidylglycerol synthase (A-PGS).

The A-PGS protein from *P. aeruginosa* shares 22 % amino acid sequence identity with MprF from *S. aureus*. In the C-terminal protein region a high conservation (35 % identity, 52 % homology) of amino acid residues was observed, whereas the N-terminal region was less conserved. A complete sequence alignment can be found in the appendix.

Using the program PSORTb v 2.0 the subcellular localization of the ORF PA0920 gene product was predicted in the cytoplasmic membrane with the highest possible score. Furthermore, no cleavable N-terminal signal peptide was proposed (Gardy *et al.*, 2005).

Prediction of protein domains of A-PGS by the program TMPred (Hofmann and Stoffel, 1993) revealed a two domain protein architecture (Fig. 7).

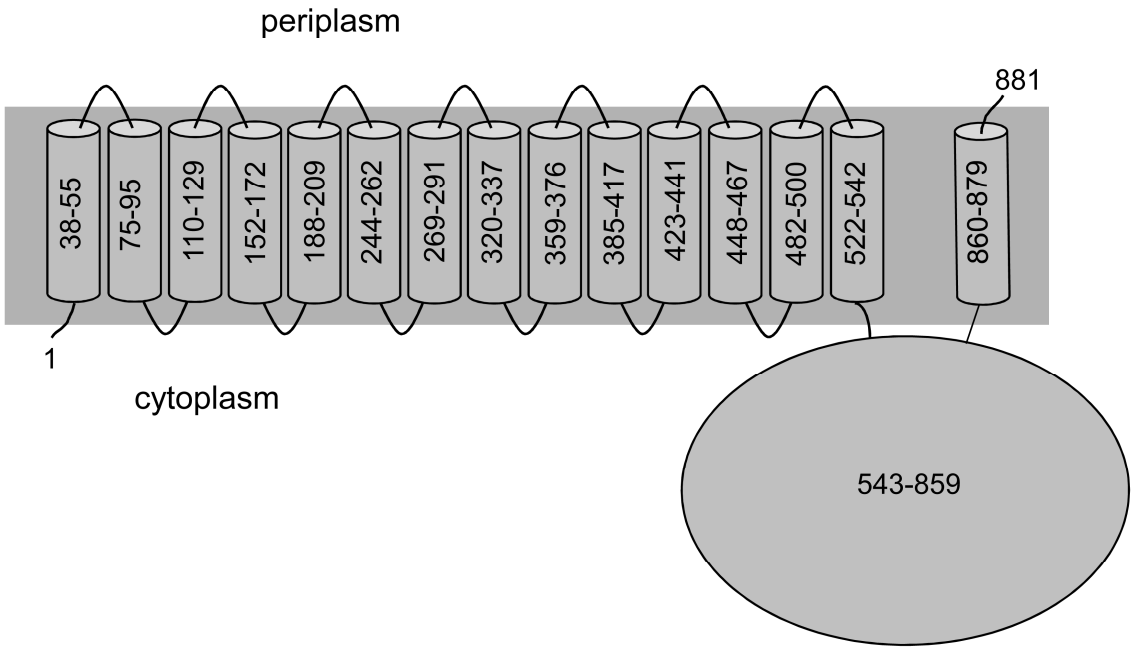



**Figure 7: Protein architecture of A-PGS from *P. aeruginosa* predicted by TMPred.**
The A-PGS protein sequence was subjected to TMPred prediction (Hofmann and Stoffel, 1993). Based on the prediction the pointed domain model was designed. Numbers specify the amino acid residues.

The A-PGS protein from *P. aeruginosa* was proposed to possess an N-terminal transmembrane domain consisting of 14 transmembrane helices (amino acid residues 1 - 542) followed by a C-terminal more hydrophilic domain containing an additional putative transmembrane helix (residues 543-881) (Fig. 7). According to this theoretical analysis, the N-terminus of the protein is located at the cytoplasmic site of the membrane, whereas the C-terminus is located in the periplasm. Furthermore, comparison of A-PGS with protein families using `Pfam protein families database` revealed similarities to an uncharacterized protein family (UPF0104) and an uncharacterized conserved protein (DUF2156) (Finn *et al.*, 2010).

## 3.2 A-PG Synthesis in *Pseudomonas aeruginosa*

### 3.2.1 A-PG Formation in *Pseudomonas aeruginosa*

Overproduction of A-PGS from *P. aeruginosa* in *E. coli* resulted in the synthesis of an amino group containing phospholipid (Lorenzo, 2006; Klein, 2007). However, the lipid composition of wild type *P. aeruginosa* cells cultivated in LB medium, in AB minimal medium or in magnesium/glucose depleted medium (Kenward *et al.*, 1979) revealed no detectable A-PG synthesis in lipid analysis *via* 2D-TLC and molybdatophosphoric acid staining (Piekarski, 2007). Therefore, formation of A-PG in *P. aeruginosa* was elucidated using radioactively labeled L-alanine. For this purpose, *P. aeruginosa* wild type and *P. aeruginosa* ΔPA0920 deletion mutant were cultivated in LB medium to an $OD_{578}$ of 1.6 and disrupted by a passage through a French® Press. This crude cellular extract was combined in an *in vivo* activity assay with 2 mM ATP, an ATP-regenerating system and 44 µM [U-$^{14}$C]-L-alanine and incubated for 1 h at 37 °C. Subsequently, extracted lipids were separated by 2D-TLC and radioactively labeled phospholipids were detected by autoradiography. Figure 8 *A* shows the autoradiography analysis of the lipid composition of *P. aeruginosa* wild type extract. In Figure 8 *B* and *C* a cellular extract of the *P. aeruginosa* ΔPA0920 deletion mutant or alternatively the *P. aeruginosa* wild type extract pre-treated with RNase A was employed.

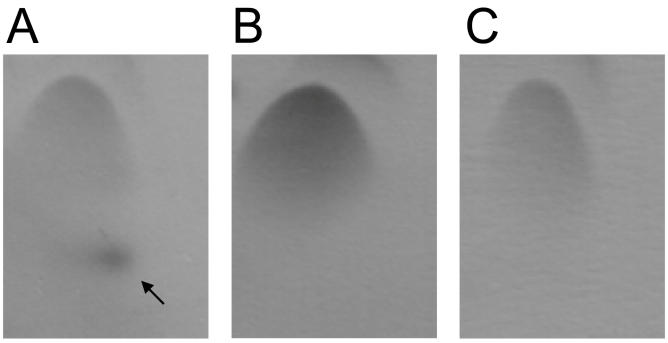



**Figure 8: Synthesis of A-PG by *P. aeruginosa* wild type and ΔPA0920 deletion mutant.**
*P. aeruginosa* wild type and *P. aeruginosa* ΔPA0920 deletion mutant were cultivated in LB medium to an $OD_{578}$ of 1.6. Cells were harvested, suspended in lysis buffer 1 (chapter 2.6.2) and disrupted by a passage through a French® Press. Obtained crude cellular extracts were subjected to an i*n vivo* assays with [U-$^{14}$C]-L-alanine as described in "Materials and Methods". After incubation of the assay for 1 h at 37 °C, lipids were extracted, subjected to 2D-TLC analysis and $^{14}$C-phospholipids were visualized by autoradiography. *A*, Lipid composition of crude cellular extract of *P. aeruginosa* wild type. *B*, Lipid composition of crude cellular extract of *P. aeruginosa* ΔPA0920 deletion mutant. *C*, Lipid composition of crude cellular extract of *P. aeruginosa* wild type, pre-treated with RNase A (100 µg ml$^{-1}$) for 25 min. A-PG is indicated by an *arrow*.

The analysis of the lipid composition of *P. aeruginosa* wild type clearly indicates the formation of a radioactively labeled lipid spot (Fig. 8, *A*). This spot was identical in position and shape to A-PG which was found in A-PGS overproducing *E. coli* (Lorenzo, 2006; Klein, 2007; Piekarski, 2007). The *P. aeruginosa* ΔPA0920 deletion mutant lacks the spot for A-PG (Fig. 8, *B*). Pre-treatment of *P. aeruginosa* wild type crude cellular extract with RNase A abolished detectable A-PG synthesis (Fig. 8, *C*), indicating the involvement of a tRNA substrate. The tRNA involvement of A-PG and L-PG formation was recently described for *S. aureus* MprF, *B. subtilis* MprF and *C. perfringens* MprF1 and MprF2 (Staubitz *et al.*, 2004; Roy and Ibba, 2008a; Roy and Ibba, 2009).
From these results it was concluded that ORF PA0920 codes for a functional gene which is responsible for A-PG synthesis in *P. aeruginosa*.

### 3.2.2 A-PG Synthesis in *P. aeruginosa* under Acidic Growth Conditions

In *S. aureus* and *E. faecalis* lowering the pH of the cultivation medium resulted in increasing amounts of the PG-derivative L-PG (Houtsmuller and van Deenen, 1965; Gould and Lennarz, 1970). To investigate a possible pH dependent A-PG synthesis in *P. aeruginosa*, acidic and neutral media were used for cultivation. To analyze acidic

growth conditions a pH of 5.3 was chosen. At this pH value only a slightly diminished growth was observed when compared to neutral growth conditions (pH 7.3).

For comparison of the lipid composition, *P. aeruginosa* wild type, ΔPA0920 and the chromosomal complementation of ΔPA0920 (ΔPA0920compl) were cultivated in AB minimal medium and LB medium at neutral and acidic pH values, respectively. The extracted lipids were separated by 2D-TLC and visualized by molybdatophosphoric acid as outlined before (Fig. 9).

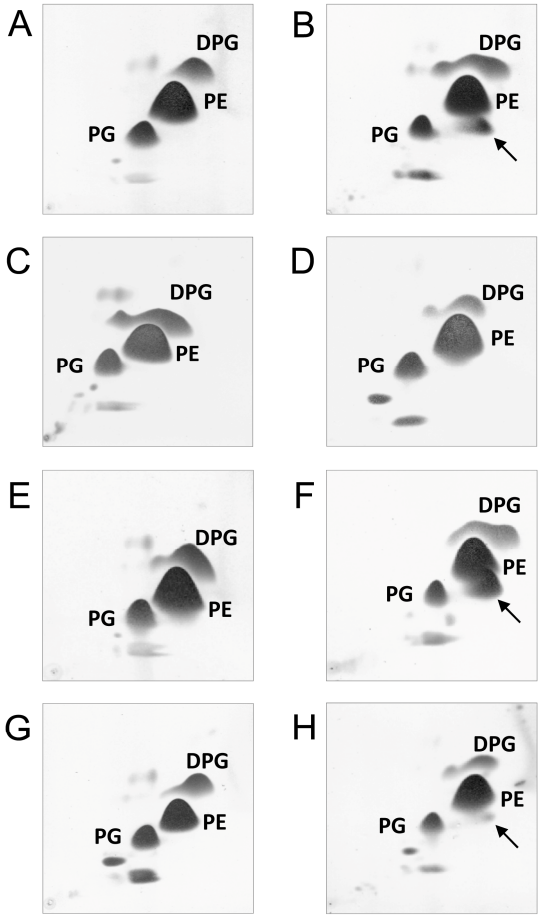



**Figure 9: Lipid composition of *P. aeruginosa* strains under neutral and acidic growth conditions.** Cells were cultivated in neutral and acidic AB minimal medium and LB medium for 24 h at 37 °C and 180 rpm as described in "Materials and Methods". After cultivation, lipids were extracted, separated by 2D-TLC and visualized by spraying with 5 % (w/v) molybdatophosphoric acid. The following strains and conditions were employed: Lipid composition of *P. aeruginosa* wild type grown in AB minimal medium with pH 7.3 (*A*) and pH 5.3 (*B*). *P. aeruginosa* ΔPA0920 deletion mutant cultivated in AB minimal medium with pH 7.3 (*C*) and pH 5.3 (*D*). Chromosomal complementation of *P. aeruginosa* ΔPA0920 with the gene encoding A-PGS (ΔPA0920compl) grown in AB minimal medium with pH 7.3 (*E*) and pH 5.3 (*F*). *P. aeruginosa* wild type cultivated in LB media with a pH of 7 (*G*) and a pH of 5.5 (*H*). The pH value of cultivation medium remained constant over time. A-PG is indicated by *arrows*. PE = phosphatidylethanolamine, PG = phosphatidylglycerol, DPG = diphosphatidylglycerol.

The analysis of lipid composition by 2D-TLC using molybdatophosphoric acid staining revealed the occurrence of an additional lipid spot for *P. aeruginosa* wild type cultivated under acidic conditions in AB minimal medium and LB medium (Fig. 9, *B, H*). This spot was also stained positive with Molybdenum Blue and ninhydrin-reagents indicating an amino group containing phospholipid (data not shown). The additional phospholipid represented approximately 6 % (AB medium) or 1 % (LB medium) of the overall *P. aeruginosa* lipid composition as judged by integration of spot intensities (data not shown). Due to running behavior similar to the additional lipid synthesized after overproduction of A-PGS in *E. coli* in 2D-TLC analysis it was assumed that this lipid represents A-PG. Under neutral growth conditions no A-PG formation was detectable by staining with 5 % (w/v) molybdatophosphoric acid (Fig. 9, *A, G*). When *P. aeruginosa* was cultivated in AB medium under alkaline conditions (pH 9.5), no A-PG synthesis was observed (data not shown). Furthermore, cultivation of *P. aeruginosa* ΔPA0920 deletion mutant under acidic and neutral conditions in AB minimal medium (Fig. 9, *C, D*) and LB medium (data not shown) failed to induce A-PG synthesis. Consequently, inactivation of ORF PA0920 leads to a defect in A-PG synthesis. However, the complementation of *P. aeruginosa* ΔPA0920 deletion mutant with a chromosomally located ORF PA0920 with 187 bp of the upstream region and 90 bp of the downstream region (strain ΔPA0920compl) restored formation of A-PG in acidic AB medium (Fig. 9, *F*). Again, no A-PG synthesis was detected after cultivation of the complementation strain in neutral AB medium (Fig. 9, *E*), indicating the same pH-dependent regulation in the chromosomal complementation strain. Interestingly, this strain showed a slightly increased A-PG formation when compared to the wild type strain under acidic growth conditions.

These results showed a pH-dependent A-PG synthesis in *P. aeruginosa*.

### 3.2.3 A-PG Formation Successively Increase with Acidic Growth Conditions

The A-PG formation of *P. aeruginosa* wild type and ΔPA0920compl was compared at different pH values. Therefore, the pH value of AB minimal medium was successively lowered in 0.5 pH steps from 7.3 to 5.3. After cultivation, extracted lipids were separated using 2D-TLC and visualized as outlined before (Fig. 10).

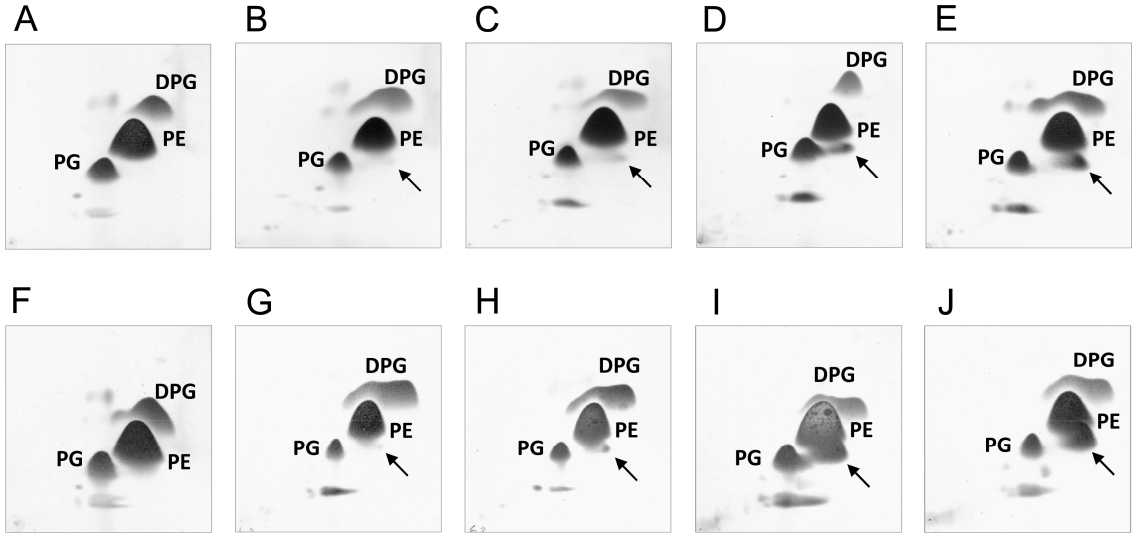



**Figure 10: Lipid composition of *P. aeruginosa* wild type and ΔPA0920compl under pH adjusted growth conditions.**
Cells were cultivated in AB minimal media for 24 h at 37 °C and 180 rpm as described in "Materials and Methods". After cultivation, lipids were extracted, separated by 2D-TLC and visualized by spraying with 5 % (w/v) molybdatophosphoric acid. Lipid composition of *P. aeruginosa* wild type (*A – E*) and of chromosomal complementation of *P. aeruginosa* ΔPA0920 (strain ΔPA0920compl) (*F – J*) cultivated in AB minimal medium are shown. The following pH values were employed: *A and F,* 7.3. *B and G,* 6.8. *C and H,* 6.3. *D and I*, 5.8. *E and J,* 5.3. The pH value of cultivation medium remained constant over time. A-PG is indicated by *arrows*. PE = phosphatidylethanolamine, PG = phosphatidylglycerol, DPG = diphosphatidylglycerol.

The lipid pattern of *P. aeruginosa* wild type and ΔPA0920compl showed a successive increase of the A-PG content when the pH value of the cultivation medium was lowered (Fig. 10, *A - E, F - J*). The relative amounts of A-PG formed in the employed media after 24 h of cultivation were determined by integration of spot intensities after analysis *via* 2D-TLC and molybdatophosphoric acid staining (Table 6).

**Table 6: Relative amounts of A-PG in *P. aeruginosa* wild type and ΔPA0920compl after cultivation in different pH adjusted medium.**
The relative amounts of A-PG related to the total amount of phospholipids formed in growth medium with different pH values after 24 h of cultivation were determined by integration of spot intensities after analysis *via* 2D-TLC and molybdatophosphoric acid staining.

| pH of AB minimal medium | relative amount of A-PG in *P. aeruginosa* wild type [%] | relative amount of A-PG in ΔPA0920compl [%] |
|---|---|---|
| 7.3 | 0.5 | 0.5 |
| 6.8 | 1 | 1 |
| 6.3 | 1 | 1 |
| 5.8 | 5 | 8 |
| 5.3 | 6 | 10 |

When *P. aeruginosa* wild type and ΔPA0920compl were cultivated in AB minimal medium with pH values of 7.3, 6.8 and 6.3 comparable amounts of A-PG (0.5, 1 and 1 %) related to the total amount of phospholipids were detected. In contrast, cultivation in AB minimal medium at pH 5.8 and 5.3 revealed an A-PG amount of 5 and 6 % for *P. aeruginosa* wild type and 8 and 10 % for *P. aeruginosa* ΔPA0920compl, respectively. These results might indicate slight differences for the regulation of the A-PG synthesis due to the genomic context of the A-PGS gene.

### 3.2.4 Biophysical Analysis of A-PG

To verify the nature of the phospholipid formed under acidic growth conditions in *P. aeruginosa*, electrospray ionization mass spectrometry (ESI-MS), gas chromatography coupled with mass spectrometry (GC/MS) and nuclear magnetic resonance (NMR) analyses were performed (Fig. 11).

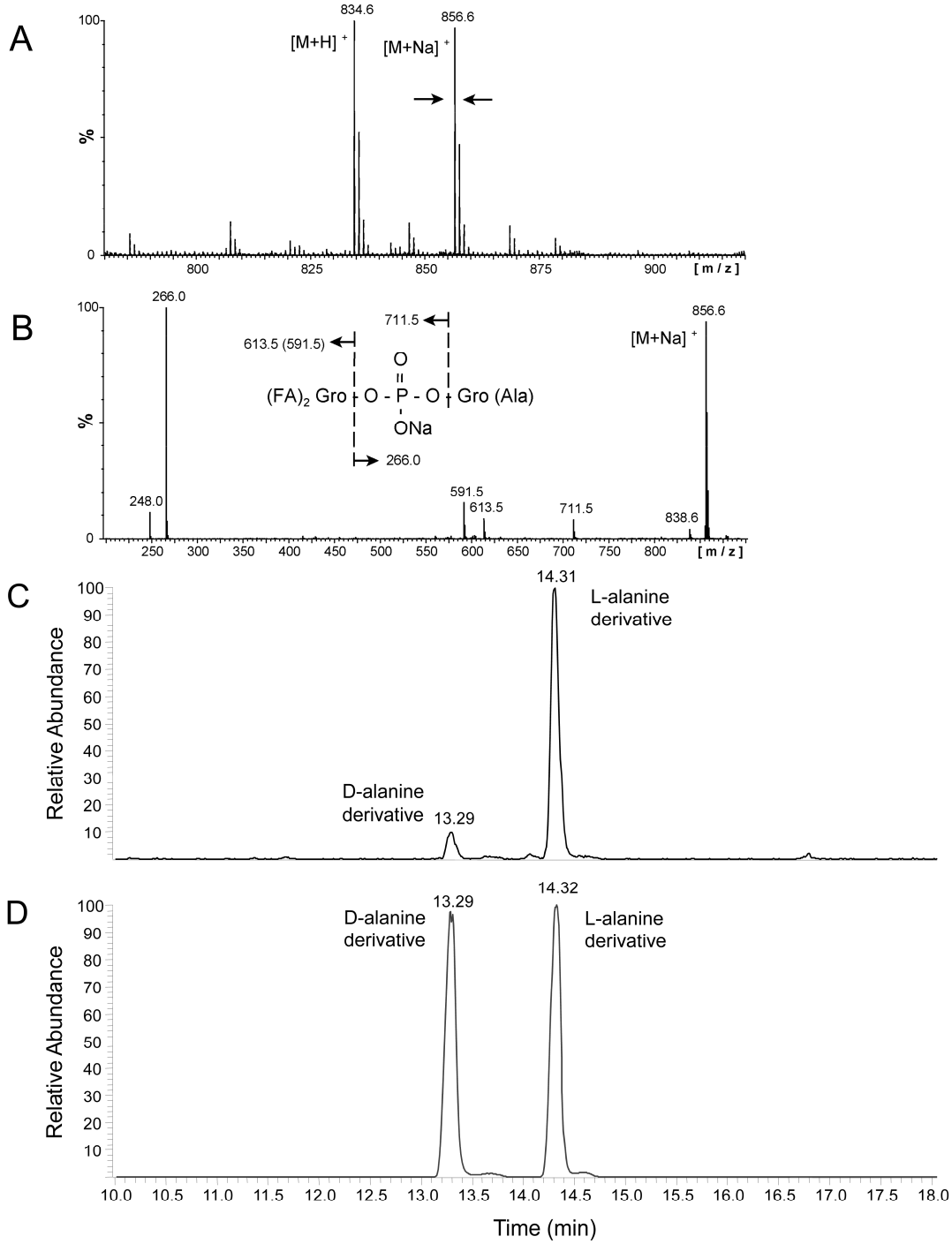



**Figure 11: ESI-MS and GC/MS analysis of the phospholipid formed under acidic growth conditions.**

*P. aeruginosa* wild type was cultivated under acidic conditions (pH 5.3) in AB minimal medium. Lipids were extracted and separated by 2D-TLC. From an unstained plate lipids were scraped, resolved and subjected to ESI-MS analysis and GC/MS analysis. *A,* Survey scan of the ESI-MS analysis. The signal at 834.6 Da corresponds to the protonated mass and the signal at 856.6 Da to the sodium salt of A-PG with C16:0 and C19:0 cis 9,10 cyclopropane fatty acid residues. *B*, Daughter ion spectrum of the molecular ion at 856.6 Da. Detected ions are compatible with the proposed structure as shown in the inserted fragmentation scheme. *C,* GC/MS spectrum of D- and L-alanine derivative derived from hydrolysis and derivatization with isopropanol and pentafluoro propionyl. *D*, reference GC/MS spectrum of a racemic mixture of D- and L-alanine derivative. Gro = glycerol residue, FA = fatty acid residue.

Positive ion electrospray mass spectrometry (Fig. 11, *A*) of the lipid with molecular ions at m/z 834.6 $[M+H]^+$ and 856.6 $[M+Na]^+$ indicated the presence of a nitrogen-containing compound as deduced from the obtained uneven molecular mass. Fragments compatible with a glycerol residue bearing two fatty acid residues, which is linked by a phosphodiester bridge to a second glycerol moiety carrying an alanine residue, were detected upon collision-induced dissociation (Fig. 11, *B*).

The nature of the fatty acid residues was determined by MS/MS of the negatively charged molecular ion at m/z 832.6 $[M-H]^-$ (data not shown). It yielded intense fragments at m/z 255.2 and 295.3. The identity of these fatty acid residues was confirmed by GC/MS analysis of the corresponding methyl esters. Authentic reference substances were used as a standard. The major fatty acids released from the intact lipid were C16:0 and C19:0 cis 9,10 cyclopropane methyl esters. These two fatty acid residues are the most abundant in *P. aeruginosa* (Hancock and Meadow, 1969). The residue linked to the second glycerol moiety was then unequivocally identified as alanine by GC/MS analysis after hydrolysis and derivatization with isopropanol and pentafluoro propionyl. Interestingly, this analysis revealed the occurrence of approximately 90 % of L-alanine and 10 % of D-alanine (Fig. 11, *C, D*). However, this result is in conflict with the proposed tRNA-dependent mechanism of A-PG synthesis. It was hypothesized that a partial racemization takes place during A-PG synthesis. Interestingly, the occurrence of D-alanyl-phosphatidylglycerol was only described for *Mycoplasma laidlawii* strain B at a molar ratio of 2:1 for D-alanyl-phosphatidylglycerol and L-alanyl-phosphatidylglycerol (Koostra and Smith, 1969).

Subsequently, NMR spectroscopy was used to identify the linkage position of the alanine residue to the second glycerol moiety (data not shown). Due to the limited amounts of material only a one dimensional $^1H$ spectrum was recorded. The obtained spectrum was compared with the $^1H$ spectrum of a derivative without an attached alanine moiety in order to identify the signals for the two glycerol units. Two characteristic broad multiplet resonances compatible with centrally acylated glycerol residues at 5.22 and 5.27 p.p.m. were detected. A well-resolved double doublet at 3.55 p.p.m. with couplings of 11.5 and 6 Hz corresponded to one proton of a non-acylated $CH_2$-OH group adjacent to an asymmetric centre (data not shown). Consequently, alanine must be linked to the central carbon of the second glycerol

moiety. This assignment is in good agreement with the central linkage position for various amino acids to compounds of the DPG class (Fischer and Leopold, 1999; Thedieck *et al.*, 2006). These results suggest an identical binding position for amino acids to asymmetric phosphatidylglycerol compounds as usually found for the symmetric counterparts, which has never been unequivocally determined prior to this work.

## 3.3 Transcriptional Analysis of ORF PA0920 Encoding A-PGS

### 3.3.1 The A-PGS Gene Promoter is Induced under Acidic Conditions

To elucidate the regulatory mechanism of A-PG formation, the regulation of the A-PGS gene promoter was studied under various growth conditions with the chromosomal transcriptional promoter–*lacZ* reporter gene fusion PAO1-$P_{PA0920}$-*lacZ*. This strain contains a 469 bp fragment of the upstream region of the A-PGS gene. The PAO1-$P_{PA0920}$-*lacZ* was cultivated under different pH conditions in AB minimal medium and LB medium, respectively. At different growth phases β-galactosidase activities were determined (Fig. 12).

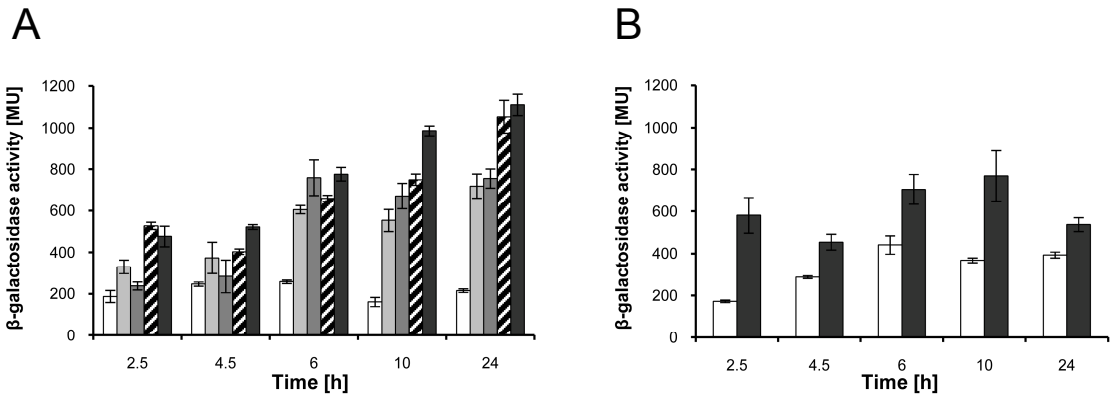



**Figure 12: Control of the A-PGS gene promoter by the pH value of the growth medium.**
Cells were cultivated in AB minimal medium or LB medium for 24 h at 37 °C and 200 rpm as described in "Materials and Methods". The results of $P_{PA0920}$–*lacZ* reporter gene fusion experiments in *P. aeruginosa* wild type (strain PAO1-$P_{PA0920}$-*lacZ*) during cultivation in different pH-adjusted media are shown. *A,* Cultivation in AB minimal medium. The following conditions were employed: pH 7.3, *white bars*. pH 6.8, *light grey bars*. pH 6.3, *grey bars*. 5.8, *striped bars*. pH 5.3, *black bars*. *B,* Cultivation in LB medium. The following conditions were employed: pH 7, *white bars*. pH 5.5, *black bars*. The β-galactosidase activities were determined at indicated time points. All experiments were repeated three times. Standard deviations are indicated.

The promoter activity in cells grown in AB medium at pH 7.3 (Fig. 12, *A white bars*) remained constant at approximately 250 MU. However, lowering of pH values to 6.8 and 6.3 resulted in an increase of β-galactosidase activity up to 718 ± 58 MU (Fig. 12, *A light grey bars*) and 755 ± 46 MU (Fig. 12, *A grey bars*), respectively, and further to 1053 ± 78 MU (Fig. 12, *A striped bars*) and 1110 ± 52 MU (Fig. 12, *A black bars*) for cultivation of the reporter strain at pH 5.8 and 5.3 for 24 h, respectively.

A comparable activation of the A-PGS gene promoter was also detected in LB medium with a pH value of 5.5 (Fig. 12, *B*). The promoter activity in cells grown in LB medium at pH 7 only slightly increased up to 441 ± 44 MU (6 h) (Fig. 12, *B white bars*). Cultivation in acidic LB medium (pH 5.5) resulted in a β-galactosidase activity up to 770 ± 120 MU after 10 h of cultivation (Fig. 12, *B black bars*). However, at the beginning of the stationary growth phase (10 h), only a slight 2-fold increase of the observed promoter activity was measured for cultivation in LB medium, whereas a 5-fold increase was determined for cultivation in AB medium under acidic conditions.

These results provide evidence for a pH-controlled promoter located in the region 469 bp upstream of ORF PA0920. Since the observed promoter activities were also dependent on the employed culture medium, one might expect that additional stimuli are mediated.

### 3.3.2 General Stress Response Regulator RpoS and Stringent Response Regulators SpoT and RelA are Not Involved in Promoter Induction of ORF PA0920

The alternative sigma factor RpoS functions as a regulator of the general stress response. *P. aeruginosa rpoS* mutants show an increased susceptibility to carbon starvation, heat shock, increased osmolarity, low pH and hydrogen peroxide (Schuster *et al.*, 2004). The *relA* and *spoT* genes are regulatory components of the stringent response (Mittenhuber, 2001). Chromosomal transcriptional promoter–*lacZ* reporter gene fusions for PA0920 in the *rpoS* deficient mutant strain PAO-MW20 (Whiteley *et al.*, 2000) and in the *spoT*/*relA* deficient mutant strain NB170 (Boes *et al.*, 2008) were constructed (PAO-SK03 and PAO-SK04) and the β-galactosidase activities of these strains were determined (Fig. 13).

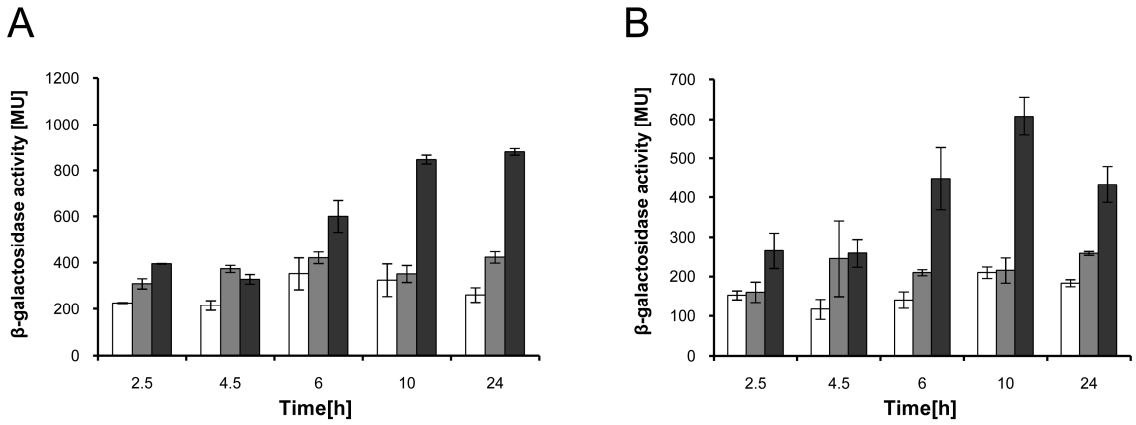



**Figure 13: Control of the A-PGS gene promoter by the pH value of the growth medium in *P. aeruginosa rpoS* mutant PAO-MW20 and in *spoT/relA* deficient mutant strain NB170.**
Cells were cultivated in AB minimal media for 24 h at 37 °C and 200 rpm as described in "Materials and Methods". The results of $P_{PA0920}$-*lacZ* reporter gene fusion experiments in *P. aeruginosa* PAO-MW20 (strain PAO-SK03) (*A*) and in NB170 (strain PAO-SK04) (*B*) during cultivation in different pH-adjusted AB minimal medium are shown. The following conditions were employed: pH 7.3, *white bars*. pH 6.3, *grey bars*. pH 5.3, *black bars*. The β-galactosidase activities were determined at indicated time points. All experiments were repeated three times. Standard deviations are indicated.

The A-PGS promoter induction for the *rpoS* deficient mutant PAO-MW20 (strain PAO-SK03) and the *spoT*/*relA* deficient mutant NB170 (strain PAO-SK04) was comparable to the induction pattern obtained for PAO1-$P_{PA0920}$-*lacZ* under various pH conditions (Fig. 13). Neither the general stress response regulator RpoS (Fig. 13, *A*) nor the stringent response regulators SpoT and RelA (Fig. 13, *B*) mediated pH-induced transcription of $P_{PA0920}$.

Furthermore, to underline these observations the lipid composition of *rpoS* and *spoT*/*relA* mutant under acidic and neutral growth conditions were elucidated (Fig. 14).

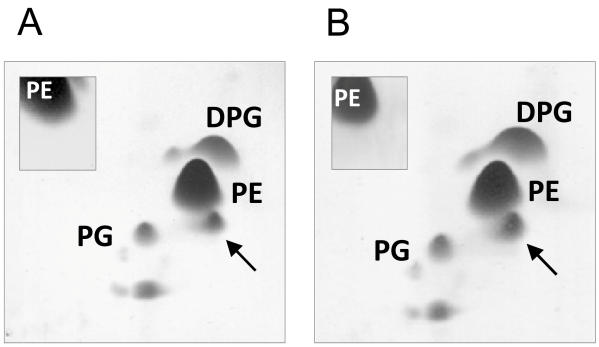



**Figure 14: Lipid composition of *rpoS* deficient mutant strain PAO-MW20 and *spoT*/*relA* deficient mutant strain NB170 under neutral and acidic growth conditions.**
Cells were cultivated in AB minimal medium for 24 h at 37 °C and 180 rpm as described in "Materials and Methods". After cultivation, lipids were extracted, separated by 2D-TLC and visualized by spraying with 5 % (w/v) molybdatophosphoric acid. *A*, Lipid composition of *rpoS* deficient mutant strain PAO-MW20 at pH 5.3 and pH 7.3 (*inlet*). *B*, Lipid composition of *spoT*/*relA* deficient mutant strain NB170 at pH 5.3 and pH 7.3 (*inlet*). A-PG is indicated by *arrows*. PE = phosphatidylethanolamine. PG = phosphatidylglycerol. DPG = diphosphatidylglycerol.

When *rpoS* and *spoT*/*relA* mutants were cultivated under neutral growth conditions (pH 7.3) no A-PG formation was detected (Fig. 14, *A, B inlets*), whereas cultivation in acidic growth medium clearly resulted in the formation of A-PG (Fig. 14, *A, B*). Furthermore, this 2D-TLC analysis revealed that both investigated *P. aeruginosa* mutant strains produce equal amounts of A-PG under acidic growth conditions as observed for the *P. aeruginosa* wild type (compare Fig. 9).
Therefore, an influence on the A-PGS promoter activity and on A-PG formation based on regulators RpoS and SpoT/RelA was ruled out.

## 3.4 Phenotypical Characterization of *P. aeruginosa* ΔPA0920 Deletion Mutant

A standard phenotypical characterization as well as the high-troughput Biolog® phenotype microarray system was used to get further insights into the biological role of A-PGS catalysis.

### 3.4.1 Standard Phenotypical Characterization

Standard phenotype assays were employed to investigate, if the *P. aeruginosa* ΔPA0920 deletion mutant is impaired in biofilm formation, in motility or in the excretion of extracellular proteins (DNase, elastase, haemolysin, phospholipase A and C, and proteases). However, all these analyses revealed no differences in comparison to *P. aeruginosa* wild type (data not shown).

Moreover, identical growth curves for the *P. aeruginosa* ΔPA0920 deletion mutant and the *P. aeruginosa* wild type strain were obtained under acidic and neutral pH conditions (data not shown), indicating no direct relevance of A-PG for the growth behavior.

It was proposed before, that the synthesis of A-PG in *P. aeruginosa* might be a prerequisite for stress adaptation as a response to changing environmental conditions, for example under the investigated acidic conditions. Analogous acid-dependent synthesis of L-PG was observed for *S. aureus* and *E. faecalis*, but the precise functional role of this lipid modification was not elucidated to date (Houtsmuller and van Deenen, 1965; Gould and Lennarz, 1970).

However, such acid tolerance mechanisms were observed in the Gram-negative bacterium *Sinorhizobium medicae*. A mutant which lacks the *mprF* homolog gene *lpiA* (*low-pH inducible gene A*) was compromised in its ability to survive acidic conditions. Interestingly, the amount of aminoacylated PG in *S. medicae* wild type grown under acidic growth conditions was below the detection limit of the employed assay (Reeve *et al.*, 2006). This might indicate that even a small amount of aa-PG can dramatically influence and modify the composition and biophysical parameters of the membrane. In future experiments a possible influence of A-PG containing membranes in acid tolerance mechanisms of *P. aeruginosa* has to be investigated.

### 3.4.2 High-Troughput Biolog® Phenotype Microarray System

To further analyze the sophisticated phenotype of the A-PG deficient *P. aeruginosa* ΔPA0920 deletion mutant a total of 1152 different culture conditions were tested in a phenotype microarray (Biolog, Hayward, CA). The employed test system is based on the reduction of a tetrazolium dye to a purple formazan in the presence of NADH in the cell. The formation of the purple formazan can be photometrically detected. The analysis revealed a set of 38 conditions, in which the *P. aeruginosa* wild type strain and the *P. aeruginosa* ΔPA0920 deletion mutant showed phenotypical differences. In a second set of experiments the individual conditions of the commercial phenotype array were independently reproduced by mixing the components in 96-well microplates thereby varying the individual concentrations of the media constituents. The corresponding phenotypic changes of *P. aeruginosa* strains wild type, ΔPA0920 and ΔPA0920compl were determined by measuring absorption of the reduced dye at 600 nm at defined time points. The *P. aeruginosa* ΔPA0920 deletion mutant showed phenotypic response due to $CrCl_3$, protamine sulphate, sodium lactate and cefsulodin in the growth medium.

#### 3.4.2.1 Toxic Effects of $Cr^{3+}$ Addition

*P. aeruginosa* wild type and ΔPA0920compl in comparison to ΔPA0920 deletion mutant showed clear differences in the metabolic activity when $CrCl_3$ was added. Cultivation in the presence of 3.5 mM $CrCl_3$ resulted in an $A_{600}$ of 0.17 for *P. aeruginosa* wild type and ΔPA0920compl, whereas the ΔPA0920 deletion mutant revealed a significantly lower absorption of 0.05 (Fig. 15).

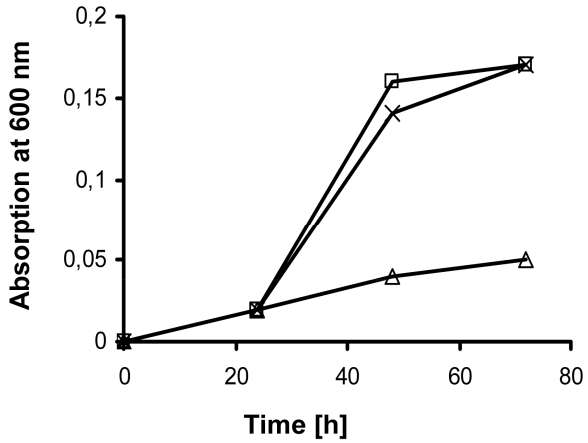



**Figure 15: Response of *P. aeruginosa* wild type, ΔPA0920 deletion mutant and ΔPA0920compl to 3.5 mM of $CrCl_3$.**
The tetrazolium dye reduction-dependent color development of cultures treated with $CrCl_3$ is shown. *P. aeruginosa* wild type (*squares*), ΔPA0920 deletion mutant (*triangles*), and the complemented ΔPA0920 deletion mutant (strain ΔPA0920compl) (*crosses*) were mixed with 3.5 mM of $CrCl_3$ in a total volume of 240 µl IF medium (Biolog) and incubated in 96-well plates. At different time points, the $A_{600}$ was measured using a Fusion™ plate reader (Perkin Elmer). For each condition, one representative experiment out of two independent experiments is shown.

$Cr^{3+}$ is a heavy metal ion generating toxic effects by its ability to bind to phosphate residues of DNA and RNA (Kortenkamp and O'Brien, 1991; Bridgewater *et al.*, 1994). Furthermore, additional toxic effects due to the ability of $Cr^{3+}$ to bind to carboxyl and sulphydryl groups in proteins have been reported (Levis and Bianchi, 1982).
The *E. coli* membrane system was shown to be a very efficient barrier towards $Cr^{3+}$ when compared with other toxic Cr(IV) species (Yao *et al.*, 2008). However, cationic Cr(III) derivatives bind tightly to *B. subtilis* and *E. coli* cell walls (Flemming *et al.*, 1990). Therefore, one might conclude that the membrane of the A-PG deficient ΔPA0920 deletion mutant is more susceptible to $Cr^{3+}$ or that the binding capacity of the membrane for $Cr^{3+}$ is changed compared to the *P. aeruginosa* wild type.

### 3.4.2.2 A-PGS-Related Resistance to the Cationic Peptide Protamine

Addition of protamine sulphate resulted into different metabolic activities for *P. aeruginosa* wild type and the complementation strain ΔPA0920compl in comparison to ΔPA0920 deletion mutant. Cultivation in the presence of 5.6 mM protamine sulphate led to $A_{600}$ values of 0.29 for the *P. aeruginosa* wild type strain and of 0.36 for ΔPA0920compl. In contrast, the ΔPA0920 deletion mutant only showed an $A_{600}$ of 0.02 after 72 h (Fig. 16).

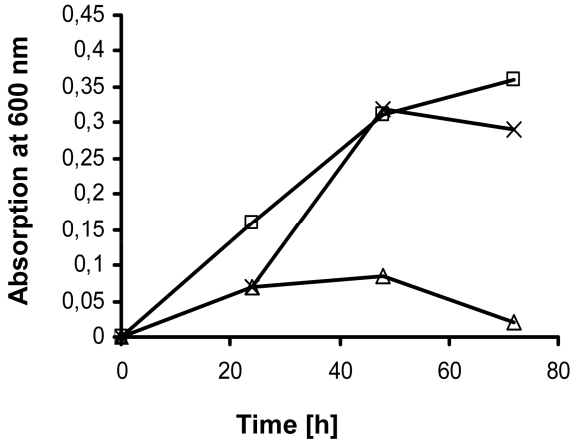



**Figure 16: Response of *P. aeruginosa* wild type, ΔPA0920 deletion mutant and ΔPA0920compl to 5.6 mM of protamine sulphate.**
The tetrazolium dye reduction-dependent color development of cultures treated with protamine sulphate is shown. *P. aeruginosa* wild type (*squares*), ΔPA0920 deletion mutant (*triangles*), and the complemented ΔPA0920 deletion mutant (strain ΔPA0920compl) (*crosses*) were mixed with 5.6 mM of protamine sulphate in a total volume of 240 µl IF medium (Biolog) and incubated in 96-well plates. At different time points, the $A_{600}$ was measured using a Fusion™ plate reader (Perkin Elmer). For each condition, one representative experiment out of two independent experiments is shown.

Protamine is a highly cationic peptide showing antimicrobial activity against a whole range of Gram-positive and Gram-negative bacteria (Johansen *et al.*, 1997). Its activity is believed to be dependent on the electrostatic attraction between the positively charged molecule and the negatively charged cell envelope, thereby resulting in disruption of the cell membrane and cell wall. Appropriate concentrations cause an inhibition of cell growth and subsequently lead to cell death due to the leakage of intracellular components including $H^+$, ATP and intracellular enzymes by pore formation (Johansen *et al.*, 1997; Toke, 2005). A comparable resistance to CAMPs in response to the lysinylation of PG or DPG has been described for *S. aureus* and for *L. monocytogenes* (Peschel *et al.*, 2001; Thedieck *et al.*, 2006). However, these related lipid alterations

resulted in an overall net cationic charge of the modified phospholipid molecule due to the lysine addition. Efficient repulsion of CAMPs due to membrane-localized L-PG was concluded (Peschel *et al.*, 2001; Staubitz *et al.*, 2004).

Recently, determination of transposon insertion sites in protamine-hypersusceptible mutants of *B. anthracis* revealed that in all mutants the ORF BA1486 was affected which encodes an orthologous MprF protein (Samant *et al.*, 2009).

The modification of PG with alanine in *P. aeruginosa* produces the zwitter ionic lipid A-PG carrying no overall net charge compared to modification with lysine which results into a positive net charge. Nevertheless, clear resistance to the cationic peptide protamine is mediated arguing against a simple "repulsion mechanism" for *P. aeruginosa*. Since, A-PG formation in *P. aeruginosa* is also involved in CAMP resistance, it is presumed that two different enzymes have evolved allowing for a more specific adaptation to the particular environment. However, to date it is not clear whether lysinylation of PG, alanylation of PG but also the modification with other amino acids have a comparable effect on the physiology of the organism.

One might conclude that A-PG formation results in a diminished binding capacity for CAMPs, thus hampering the initial step of antimicrobial action. Besides this, the subsequent interaction of protamine with the overall bilayer is of importance. A-PG incorporation might alter the overall membrane permeability of *P. aeruginosa* thereby preventing the disruption of the membrane structure due to the incorporation of cationic peptide molecules.

#### 3.4.2.3 A-PGS-Mediated Resistance to the Osmolyte Sodium Lactate

*P. aeruginosa* wild type and ΔPA0920compl in comparison to ΔPA0920 deletion mutant showed clear differences in the metabolic activity when sodium lactate was added. The presence of 625 mM sodium lactate resulted in a final $A_{600}$ of 0.09 for the *P. aeruginosa* wild type strain, 0.15 for the ΔPA0920compl strain and 0.02 for the ΔPA0920 deletion mutant after 72 h (Fig. 17).

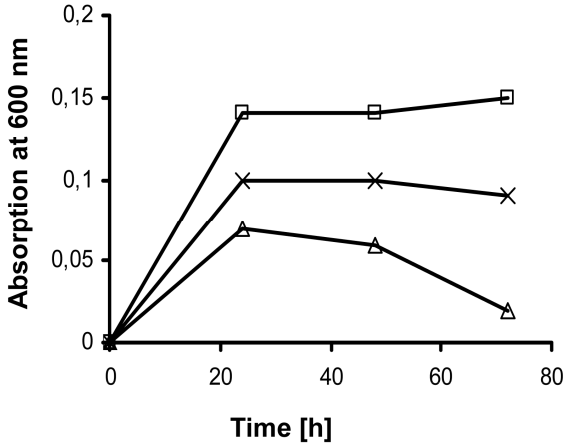



**Figure 17: Response of *P. aeruginosa* wild type, ΔPA0920 deletion mutant and ΔPA0920compl to 625 mM of sodium lactate.**
The tetrazolium dye reduction-dependent color development of cultures treated with sodium lactate is shown. *P. aeruginosa* wild type (*squares*), ΔPA0920 deletion mutant (*triangles*), and the complemented ΔPA0920 deletion mutant (strain ΔPA0920compl) (*crosses*) were mixed with 625 mM of sodium lactate in a total volume of 240 µl IF medium (Biolog) and incubated in 96-well plates. At different time points, the $A_{600}$ was measured using a Fusion™ plate reader (Perkin Elmer). For each condition, one representative experiment out of two independent experiments is shown.

The capacity of microorganisms to respond to fluctuations in their osmotic environments is a fundamental physiological process that determines their abilities to survive in a variety of habitats. Therefore, it has been shown that the control of the fluidity and the permeability of the cellular membranes are essential to maintain cell homeostasis (Vanden Boom and Cronan, 1989). Highly concentrated osmolytes are frequently used as antimicrobial agents; for example, sodium lactate is employed as food supplement.

The alteration of the membrane permeability could be responsible for the specific phenotype observed for the *P. aeruginosa* mutant lacking A-PG. Furthermore, possible secondary effects might be involved in the susceptibility of the A-PG deficient mutant. As a result of high lactate concentrations outside the cell, lactic acid and protons might accumulate in the bacterial cell which in turn might result in cell damage (Rubin *et al.*, 1982; McWilliam Leitch and Stewart, 2002). A-PG incorporation in the bacterial membrane might inhibit the accumulation of lethal concentrations of lactate and protons in the cell.

### 3.2.4.4 A-PGS-Mediated Resistance to the β-Lactam Antibiotic Cefsulodin

Addition of cefsulodin resulted into different metabolic activities for *P. aeruginosa* wild type and ΔPA0920compl in comparison to ΔPA0920. The cultivation in the presence of 20 µM of cefsulodin led to an $A_{600}$ of 0.25 for the *P. aeruginosa* wild type and 0.4 for the ΔPA0920compl strain, whereas the deletion mutant only gained an $A_{600}$ of 0.06 after 72 hours (Fig. 18).

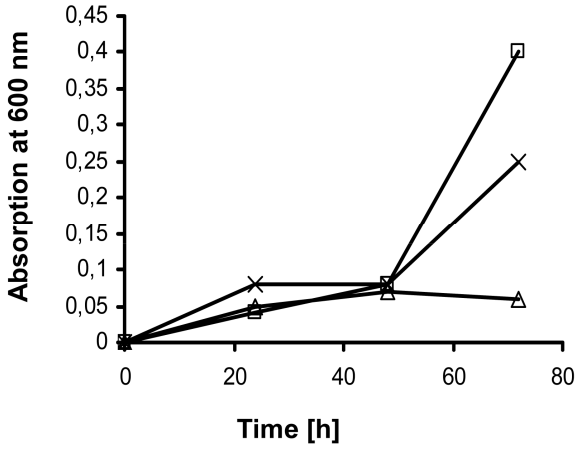



**Figure 18: Response of *P. aeruginosa* wild type, ΔPA0920 deletion mutant and ΔPA0920compl to 20 µM of cefsulodin.**
The tetrazolium dye reduction-dependent color development of cultures treated with cefsulodin is shown. *P. aeruginosa* wild type (*squares*), ΔPA0920 deletion mutant (*triangles*), and the complemented ΔPA0920 deletion mutant (strain ΔPA0920compl) (*crosses*) were mixed with 20 µM of cefsulodin in a total volume of 240 µl IF medium (Biolog) and incubated in 96-well plates. At different time points, the $A_{600}$ was measured using a Fusion™ plate reader (Perkin Elmer). For each condition, one representative experiment out of two independent experiments is shown.

Cefsulodin is a semi-synthetic cephalosporin impeding the cross-linking of peptidoglycan and therefore showing antipseudomonal activity (Tsuchiya *et al.*, 1978). The β-lactams are believed to permeate through hydrophilic pores in the outer membrane (Irvin *et al.*, 1981). Generally, *P. aeruginosa* has a high intrinsic resistance to β-lactam antibiotics due to its low outer membrane permeability and due to the presence of β-lactamases and multi-drug-efflux pumps (Lambert, 2002; Nikaido, 2003). In transposon mutagenesis studies of *S. aureus* an *mprF* mutant was found to be sensitive against the β-lactam oxacillin. Further analysis of this mutant revealed that the resistance to four different β-lactams was decreased (Komatsuzawa *et al.*, 2001).

Possibly alteration of the *P. aeruginosa* membrane systems might have an effect on the uptake of cefsulodin. Since β-lactam antibiotics do not interact directly with the lipid bilayer as they target the biosynthesis of peptidoglycan in the periplasm (Wilke *et al.*,

2005), the mode of action remains elusive. It is known, that lipid composition and the presence of certain phospholipids in the bacterial membrane might have an influence on folding and activity of membrane proteins, for example, the lactose permease LacY of *E. coli* requires phosphatidylethanolamine to obtain its native topology (Bogdanov *et al.*, 2008). According to this one might speculate that the presence of A-PG might modulate the activity of porins or other membrane proteins, which results into the observed resistance phenotypes. Interestingly, no phenotypic differences were observed for other β-lactam antibiotics tested in the Biolog® phenotype microarray system.

#### 3.2.4.5 A-PGS-dependent Homeostasis

Biological membranes play a central role in response to environmental stress. Maintenance of cell homeostasis is dependent on effective transmembrane processes, such as nutrient transport or solute gradients for energetic purposes. Current investigations clearly indicate that the distribution of phospholipids in the bacterial membrane is non-uniform (Matsumoto *et al.*, 2006). Therefore, small alterations of the lipid composition might have significant influence on the local lipid concentration. Cultivation of wild type *P. aeruginosa* in IF-10 medium and AB medium, pH 7.3 supplemented with 3.5 mM of $CrCl_3$, 5.6 mM of protamine sulphate, 625 mM of sodium lactate, and 20 µM of cefsulodin, respectively, did not result in a significantly increased A-PG content (data not shown). These results indicate that an overall A-PG content of approximately 0.5 % has an important influence on the resistance of *P. aeruginosa*. Similarly, for *S. medicae* it was recently shown that aminoacylated PG below the detection limit of the employed assay is essential for the underlying mechanism of acid tolerance (Reeve *et al.*, 2006).

From these results it becomes clear that in *P. aeruginosa* A-PGS-dependent lipid modification is a central process for the adaptation to acidic conditions. However, a total of four ‘other’ phenotypes for the *P. aeruginosa* ΔPA0920 deletion mutant indicate that synthesis of A-PG is also required under various other environmental conditions. One might expect that A-PG is able to render the binding ability for cationic molecules on the surface of the lipid bilayer. Concurrently, A-PG might also have significant

influence on the permeability and fluidity of the lipid bilayer as well as on the activity of membrane proteins.

All enzymes responsible for the modification of PG share a high degree of sequence identity, indicating the presence of a common ancestor. The different amino acid specificities might be a result of adaptation processes towards different environmental conditions. Therefore, the analysis of the biochemical and biophysical features of the different aminoacylated PGs is of importance for the understanding of bacterial resistance mechanisms.

## 3.5 Biochemical Characterization of the Catalytic Domain of the A-PGS from *Pseudomonas aeruginosa*

### 3.5.1 Cloning, Production and Purification of Truncated aa-PGS Variants

From theoretical analysis the following protein architecture of A-PGS from *P. aeruginosa* was proposed: an N-terminal transmembrane domain consisting of 14 transmembrane helices (amino acid residues 1 - 542), and a more hydrophilic C-terminal domain (543 - 881) (Fig. 7). The N-terminal domains from various organisms are highly variable in size (from 228 amino acid residues for *C. perfringens* MprF1 (Roy and Ibba, 2008b) to 542 amino acid residues for *P. aeruginosa* A-PGS) and share only an amino acid sequence identity of approximately 15 %. In contrast to this the C-terminal domains of aa-PGS can be found highly conserved with an amino acid sequence identity of approximately 30 %. Based on these theoretical findings it was concluded, that the C-terminal domain might be essential for aa-PG synthesis.

For the future analysis aa-PGS enzymes from various organisms were investigated. Therefore, the corresponding gene sequences of ORF PA0920, *mprF, lmo1695* and *lin1803* from *P. aeruginosa*, *S. aureus, L. monocytogenes* and *L. innocua*, respectively, encoding the proposed hydrophilic C-terminal domains were cloned into standard *E. coli* expression vectors (pGEX-6P-1). The resulting constructs pGEX-6P-1/PA0920ΔAS1-542, pGEX-6P-1/*mprF*ΔAS1-514, pGEX-6P-1/*lmo1695*ΔAS1-510 and pGEX-6P-1/*lin1803*ΔAS1-511, respectively, allow for the purification of truncated variants A-PGS$_{543\text{-}881}$, MprF$_{515\text{-}840}$, Lmo1695$_{511\text{-}865}$, and Lin1803$_{512\text{-}865}$ *via* a glutathione

*S*-transferase (GST) tag. Production of the corresponding proteins was performed in *E. coli* BL21 (λDE3) cells under aerobic conditions. Protein production induced by addition of 50 µM IPTG resulted in the production of significant amounts of the individual proteins from all four organisms. The purification of the GST-tagged enzymes *via* affinity chromatography with a glutathione sepharose matrix was performed according to manufacturer's instructions. Proteins were eluted with buffer containing 10 mM of glutathione. The purification of the truncated protein variants from *P. aeruginosa, S. aureus, L. monocytogenes* and *L. innocua* is summarized in Figure 19. Alternatively, the GST-tag of A-PGS$_{543\text{-}881}$ from *P. aeruginosa* was proteolytically cleaved using the PreScission Protease.

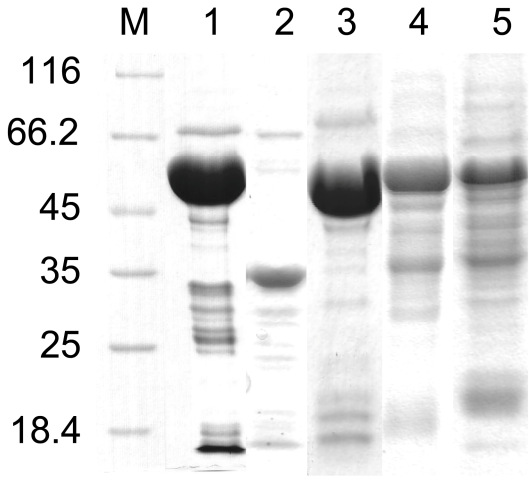



**Figure 19: SDS-PAGE analysis of purified recombinantly truncated aa-PGSs variants from different microorganisms.**
Proteins were recombinantly produced in *E. coli* BL21 (λDE3), chromatographically purified. Protein fractions were analyzed on a 12 % SDS-PAGE, and visualized *via* Coomassie Brilliant Blue staining. *Lane M*, molecular mass marker, relative molecular masses (*1'000) are indicated. *Lane 1*, purified GST-A-PGS$_{543\text{-}881}$ from *P. aeruginosa*. *Lane 2*; purified A-PGS$_{543\text{-}881}$ from *P. aeruginosa* after proteolytical cleavage of the GST-tag using the PreScission Protease. *Lane 3*, purified GST-MprF$_{515\text{-}840}$ from *S. aureus. Lane 4,* purified GST-Lmo1695$_{511\text{-}865}$ from *L. monocytogenes*. *Lane 5*, purified GST-Lin1803$_{512\text{-}865}$ from *L. innocua*.

Affinity chromatography *via* glutathione sepharose resulted in highly enriched fusion proteins. All proteins showed a slightly decreased relative molecular mass when compared to the calculated molecular mass for GST-A-PGS$_{543\text{-}881}$ from *P. aeruginosa* (*lane 1*, GST-A-PGS$_{543\text{-}881}$ = 65'132 Da). MprF$_{515\text{-}840}$ from *S. aureus* (*lane 3*, GST-MprF$_{515\text{-}840}$ = 65'371 Da), Lmo1695$_{511\text{-}865}$ from *L. monocytogenes* (*lane 4*, GST-Lmo1695$_{511\text{-}865}$ = 67'489 Da), and Lin1803$_{512\text{-}865}$ from *L. innocua* (*lane 5*,

GST-Lin1803$_{512-865}$ = 67'450 Da). After a proteolytical cleavage the observed relative molecular mass for A-PGS$_{543-881}$ from *P. aeruginosa* was in good agreement with the calculated molecular mass for the truncated protein (*lane 2*, A-PGS$_{543-881}$ = 38'719 Da).

### 3.5.2 Analytical Gel Permeation Chromatography of A-PGS$_{543-881}$

To determine the oligomeric state of A-PGS$_{543-881}$ from *P. aeruginosa*, an analytical gel permeation chromatography was performed. The following elution diagram (Fig. 20) was obtained for A-PGS$_{543-881}$ from *P. aeruginosa.*

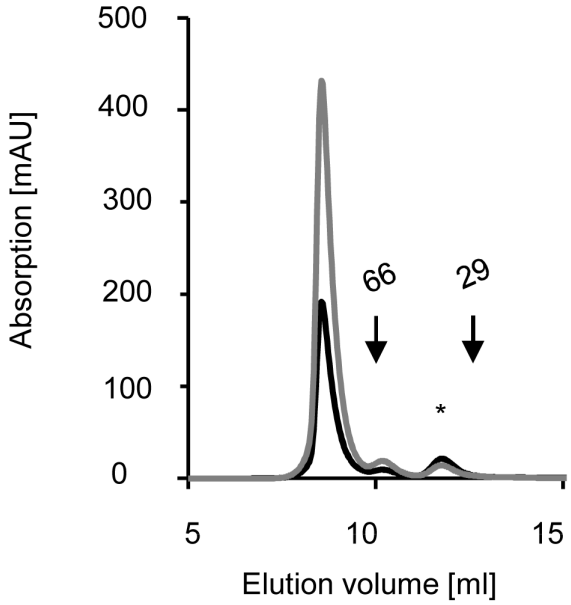



**Figure 20: Analytical gel permeation chromatography analysis of purified A-PGS$_{543-881}$ from *P. aeruginosa*.**
Purified A-PGS$_{543-881}$ after proteolytical cleavage off the GST-tag at a concentration of ~ 9 mg ml$^{-1}$ was loaded onto a Superdex 75 HR 10/30 column (GE Healthcare) and eluted with 50 mM Tris-HCl, pH 8.0, 250 mM NaCl and 2 mM DTT at a flow rate of 0.5 ml min$^{-1}$. The absorbance at 280 nm and 260 nm was monitored. Elution volumes of marker proteins (* 1'000) are marked with arrows. A-PGS$_{543-881}$ elution peak is highlighted with an *asterisk*.

The analytical gel permeation chromatography after proteolytical cleavage revealed a relative molecular mass of 34'000 for A-PGS$_{543-881}$ from *P. aeruginosa* (calculated molecular mass = 38'719 Da) indicating a monomeric soluble A-PGS$_{543-881}$ protein. In SDS-PAGE analysis of elution fractions at an elution volume of 8.61 and 10.29 ml no A-PGS$_{543-881}$ protein was detectable.

### 3.5.3 Analysis of Potential Cofactors

To identify possible cofactors of A-PGS$_{543-881}$, UV/Vis absorption spectroscopy as well as fluorescence measurements were performed. However, no evidence for a chromogenic cofactor was obtained.

## 3.6 Activity of Recombinantly Produced aa-PGS Variants

### 3.6.1 *In vivo* Activity of aa-PGS Variants

The truncated aa-PGS variants of *P. aeruginosa, L. monocytogenes, L. innocua* and *S. aureus* were tested for *in vivo* activity. For this purpose, *E. coli* BL21 (λDE3) cells heterologously producing truncated aa-PGS variants as GST fusion were harvested 3 h after induction of protein production and the total lipids of the *E. coli* host were extracted and subsequently separated by 2D-TLC (Fig. 21). This procedure allows for the identification of the *de novo* biosynthesis of aa-PG in *E. coli* under *in vivo* conditions due to the absence of any aa-PGS related genes in the host.

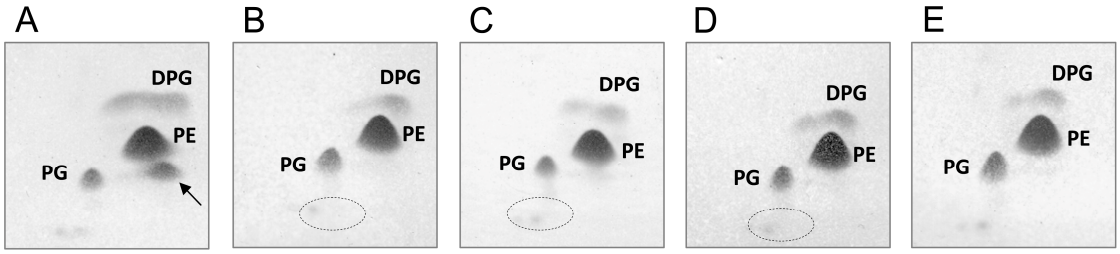



**Figure 21: Lipid composition of *E. coli* BL21 (λDE3) overproducing truncated aa-PGS variants from *P. aeruginosa*, *S. aureus*, *L. monocytogenes* and *L. innocua*, respectively.**
*E. coli* BL21 (λDE3) harboring the respective plasmid was cultivated at 37 °C and 200 rpm to an $OD_{578}$ of 0.5. Recombinant gene expression was induced by addition of 50 µM IPTG. Cells were further incubated for 3 h and harvested. Extracted lipids were separated by 2D-TLC and visualized by spraying with 5 % (w/v) molybdatophosphoric acid. *A*, *in vivo* activity of GST-A-PGS$_{543-881}$ from *P. aeruginosa*. *B, in vivo* activity of GST-MprF$_{515-840}$ from *S. aureus*. *C, in vivo* activity of GST-Lmo1695$_{511-865}$ from *L. monocytogenes*. *D, in vivo* activity of GST-Lin1803$_{512-865}$ from *L. innocua*. *E, in vivo* activity of BL21 (λDE3) carrying empty vector pGEX-6P-1 as negative control. A-PG is indicated by an *arrow*, theoretical position of L-PG is marked with an *ellipse*. PE = phosphatidylethanolamine, PG = phosphatidylglycerol, DPG = diphosphatidylglycerol.

The employed *in vivo* A-PGS activity of A-PGS$_{543-881}$ variant from *P. aeruginosa* resulted in the accumulation of approximately 10 % A-PG when compared to the total lipid content (Fig. 21, *A*). This value is higher than the observed 5 % for the overproduction of wild type A-PGS in *E. coli* TOP10 (Fig. 6) (Klein, 2007), which can be ascribed to the increased overproduction level for the truncated protein variant. The *E. coli* genome lacks genes encoding an aa-PGS. Consequently, control experiments with *E. coli* BL21 (λDE3) carrying empty vector pGEX-6P-1 as negative control showed no aa-PG synthesis (Fig. 21, *E*).

Overproduction of truncated variants MprF$_{515-840}$ from *S. aureus* (Fig. 21, *B*), Lmo1695$_{511-865}$ from *L. monocytogenes* (Fig. 21, *C*) and Lin1803$_{512-865}$ from *L. innocua* (Fig. 21, *D*) failed to result in detectable L-PGS activity. However, in a comparable *in vivo* experiment for the *S. aureus* MprF protein it was recently shown that under *in vivo* conditions 6 of the transmembrane helices are required for maintaining a functional enzyme (Ernst *et al.*, 2009). When all transmembrane helices of MprF1 from *C. perfringens* or from MprF of *B. subtilis* were deleted residual enzymatic activity was obtained under *in vitro* conditions. Besides this, no detectable lipid modification was observed with the employed *in vivo* assay (Roy and Ibba, 2009).

This initial experiment is indicative for the efficient conversion of the *E. coli* specific PG substrate by the GST-A-PGS$_{543-881}$ fusion protein under *in vivo* conditions. Furthermore, these results point out that the newly established A-PGS$_{543-881}$ protein variant from *P. aeruginosa* is an appropriate enzyme to investigate the enzymatic mechanism of A-PGS or A-PGS related systems.

### 3.6.2 *In vitro* Activity of aa-PGS Variants

In the *in vitro* aa-PGS activity assay using *E. coli* extracts the substrate molecules PG and Ala-tRNA$^{Ala}$ or Lys-tRNA$^{Lys}$ were provided by an *E. coli* crude cellular extract.

Due to the fact that truncated variants of *B. subtilis* MprF showed L-PG formation solely under *in vitro* conditions, the *in vitro* activity of truncated enzyme variants of *S. aureus*, *L. monocytogenes* and *L. innocua* was also investigated.

Therefore, 500 µl of crude cellular extract of *E. coli* overproducing the alanyl-tRNA synthetase and lysyl-tRNA synthetase, respectively, were supplemented with an ATP-regenerating system, 2 mM ATP and 20 µM of $^{14}$C labeled L-alanine or $^{14}$C labeled L-lysine. The reaction was initiated by the addition of 20 µM of each individual purified truncated aa-PGS variant. Subsequently polar lipids were extracted and characterized by 2D-TLC and autoradiography (Fig. 22).

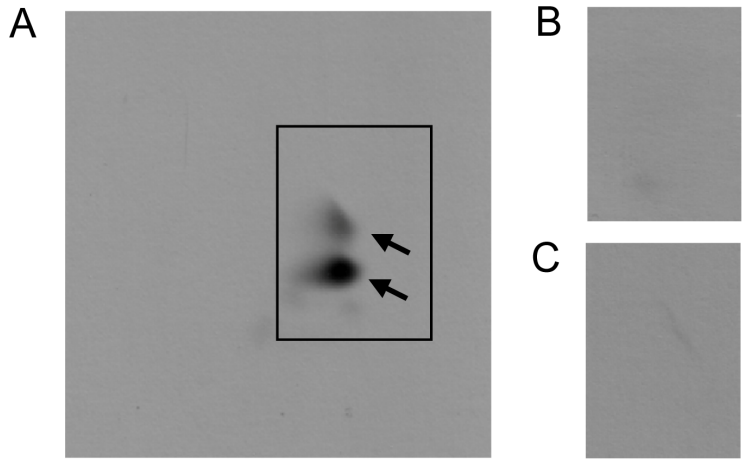



**Figure 22: Autoradiography analysis of 2D-TLC plates of *in vitro* A-PGS activity assays using *E. coli* extracts with purified truncated GST-A-PGS$_{543-881}$ variant of *P. aeruginosa*.**
*In vitro* assays using *E. coli* extracts with radioactively labeled [1-$^{14}$C]-L-alanine and 20 µM of purified GST-A-PGS$_{543-881}$ were performed as described in "Materials and Methods". After incubation of the assay for 1 h at 37 °C, lipids were extracted, subjected to 2D-TLC analysis and $^{14}$C-phospholipids were visualized by autoradiography. *A*, Activity of purified GST-A-PGS$_{543-881}$ from *P. aeruginosa*. *B,* Negative control without enzyme. *C,* Negative control, pre-treatment of crude cellular extract with 100 µg ml$^{-1}$ RNase A for 25 min. A-PG is indicated by *arrows*.

In Figure 22 the result of a typical *in vitro* assay using *E. coli* extracts for the truncated protein variant GST-A-PGS$_{543-881}$ is shown. Control experiments in the absence of GST-A-PGS$_{543-881}$ (Fig. 22, *B*) or with RNase A pre-treatment (Fig. 22, *C*) did not result in the formation of any detectable amounts of A-PG indicating a tRNA-dependent reaction mechanism. However, incubation with 20 µM of GST-A-PGS$_{543-881}$ yielded high amounts of A-PG (Fig. 22, *A*). Interestingly, the standard *in vitro* assay using *E. coli* extracts revealed two radioactively labeled spots (Fig. 22, *A arrows*). Since both of these spots are the result of A-PGS activity, one can speculate about the formation of the both isomers 3' and 2' A-PG. The analysis of 3' A-PG isomer using standard 2D-TLC procedure is hampered since this lipid spot is usually overlapped by phosphatidylethanolamine.

From these results, it was concluded, that the C-terminal domain of A-PGS (A-PGS$_{543-881}$) from *P. aeruginosa* harbors the active site. In contrast, the truncated aa-PGS variants of *S. aureus*, *L. monocytogenes and L. innocua* failed to synthesize L-PG under the employed conditions of the *in vitro* assay using *E. coli* extracts (data not shown). These results might indicate that solely the C-terminal domain of these three enzymes is not sufficient for L-PG synthesis. It was concluded that for catalytic activity additional amino acids located in the N-terminal transmembrane region are required. Based on these results A-PGS from *P. aeruginosa* was employed for all future experiments to elucidate key amino acids and the substrate requirements of the tRNA-dependent catalysis.

### 3.6.3 Quantitative Determination Method of the specific A-PGS Activity by Scintillation Counting

The detection of radioactively labeled phospholipids using autoradiography of 2D-TLC allows only for a qualitative analysis of A-PG synthesis. For the quantification of the enzymatic activity of A-PGS$_{543-881}$ [1-$^{14}$C]-L-alanine incorporation into the lipid fraction was analyzed by scintillation counting after lipid extraction. Therefore, *in vitro* assays using *E. coli* extracts with different concentrations of the purified truncated GST-A-PGS$_{543-881}$ variant of *P. aeruginosa* were performed. At different time points samples were subjected to lipid extraction and radioactively labeled phospholipids determined by scintillation counting (Fig. 23). Alternatively, solubilized full length A-PGS was used for determination of specific activity of the wild type enzyme.

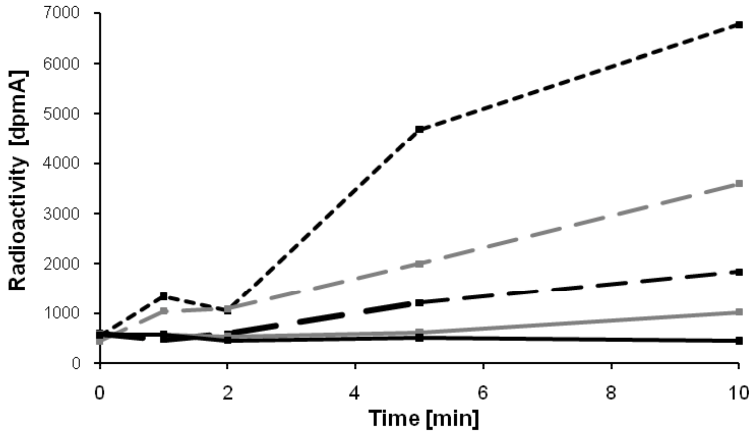



**Figure 23: Liquid scintillation analysis of *in vitro* activity of purified GST-A-PGS$_{543-881}$ from *P. aeruginosa*.**
*In vitro* assays using *E. coli* extracts with radioactively labeled [1-$^{14}$C]-L-alanine supplemented with different enzyme concentrations were performed as described in "Materials and Methods". The assay was incubated at 37 °C, at indicated time points samples of 100 µl volume were subjected to lipid extraction and liquid scintillation analysis. The following concentrations of purified *P. aeruginosa* GST-A-PGS$_{543-881}$ were employed: 0 µM (*black line*), 2 µM (*grey line*), 5 µM (*dashed black line*), 10 µM (*dashed grey line*), and 20 µM (*dotted black line*).

A linear increase of product formation using different concentrations of GST-A-PGS$_{543-881}$ from *P. aeruginosa* was observed (Fig. 23). In the presence of 2 - 10 µM of GST-A-PGS$_{543-881}$, this type of experiment resulted in a linear increase of A-PG formation over a time scale of 10 min (Fig. 23), whereas in assays in which the enzyme was omitted (*black line*) no product formation was observed. The observed activity for the *P. aeruginosa* GST-A-PGS$_{543-881}$ enzyme in the employed assay yielded a specific activity of 27 pmol · mg$^{-1}$ · min$^{-1}$ which is comparable to the measured value of 23 pmol · mg$^{-1}$ · min$^{-1}$ for the solubilized full length A-PGS protein. Interestingly, full length A-PGS and the truncated A-PGS$_{543-881}$ variant have similar product formation efficiencies indicating no critical involvement of the N-terminal domain in the catalytic mechanism.

The observed specific activity is comparable to values obtained for other membrane proteins of the phospholipid biosynthesis pathway. For example, for the phosphatidylglycerolphosphate synthase from cardiac mitochondria an activity of 15 ± 2 pmol · mg$^{-1}$ · min$^{-1}$ was observed (Cao *et al.*, 1995).

## 3.7 Substrate Specificity and Recognition of *P. aeruginosa* A-PGS - Rationale of the Approach

For modification of PG two substrate molecules - the aminoacylated tRNA and PG - have to be precisely recognized by the enzyme.

Besides protein biosynthesis the A-PGS from *P. aeruginosa* and A-PGS homolog proteins are one of the rare examples of using aminoacylated tRNA as substrate next to peptidoglycan crosslinking, synthesis of antibiotics and tetrapyrrole biosynthesis (Matsuhashi *et al.*, 1965; Yamato *et al.*, 1986; Smith *et al.*, 1992). Therefore, Ala-$tRNA^{Ala}$ has a dual function in the metabolism of *P. aeruginosa.* It is recognized by two enzymes: alanyl-tRNA synthetase (AlaRS) and A-PGS. To ensure accurate protein biosynthesis and also for the synthesis of A-PG, tRNA substrate recognition is of prime importance. Aminoacyl-tRNA synthetases distinguish their cognate tRNA by specific tRNA identity elements. The tRNA identity is affected by positive and negative elements that respectively activate specific aminoacylation and prevent false aminoacylation. The positive elements include isolated nucleotides in single-stranded regions, base pairs in helices, and can also be structural motifs. However, the anticodon bases and the discriminator base N73 are the most occurring tRNA identity elements for aminoacyl-tRNA synthetases (Crothers *et al.*, 1972; Schulman, 1991; McClain, 1993).

The substrate specificity for the amino acid part of A-PGS was determined using an *in vivo* approach in the natural host *P. aeruginosa*. The tRNA identity and the recognition elements for the PG substrate were investigated using *in vitro* activity assays. The standard A-PGS *in vitro* assay mixture contained purified PG and recombinantly purified AlaRS in the presence of a commercially available mixture of tRNAs from *E. coli.* Alternatively, the tRNA mixture was replaced with purified $tRNA^{Ala1}$ or $tRNA^{Ala2}$ from *P. aeruginosa* which were produced by *in vitro* transcription (Sampson and Uhlenbeck, 1988). Alternatively, tRNA microhelices were employed. Additionally, the PG constituent of the assay was substituted with PG derivatives. The lipid content was extracted and $^{14}C$ labeled compounds were analyzed by autoradiography after 2D-TLC. To quantify the A-PG formation in experiments with *in vitro* transcribed tRNAs or in the presence of microhelices, the polar lipids were analyzed by scintillation counting after lipid extraction from the assay mixture.

### 3.7.1 A-PGS from *P. aeruginosa* Shows a Substrate Specificity for tRNA-bound Alanine

In very early investigations the presence of L-PG in *P. aeruginosa* strain NCTC 6750 was described (Kenward *et al.*, 1979). In the present study A-PG formation for *P. aeruginosa* PAO1 was found (chapter 3.2.4). In order to unambiguously characterize the specificity of *P. aeruginosa* A-PGS, crude cellular extracts from the *P. aeruginosa* PAO1-derivative ADD1976 overproducing A-PGS were incubated with radioactively labeled alanine, lysine, and also with alanine in combination with lysine, respectively. *De novo* biosynthesis of aminoacyl-PG was monitored using 2D-TLC and autoradiography (Fig. 24).

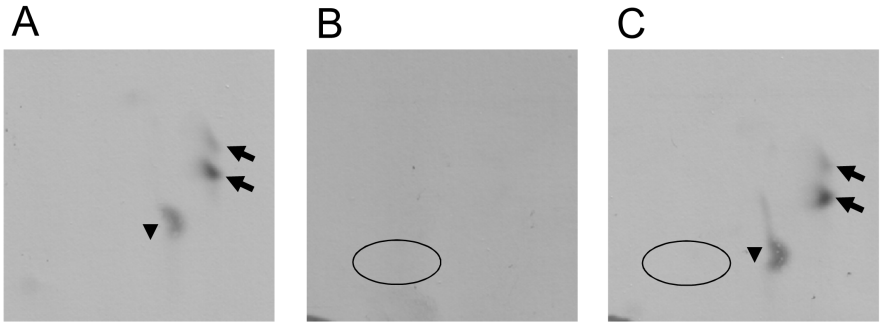



**Figure 24: *In vivo* formation of aminoacylated PG by *P. aeruginosa* crude cellular extracts.**
A 2D-TLC analysis of $^{14}$C-phospholipids is shown. Crude cellular extract from *P. aeruginosa* ADD1976 harboring pUCP20T-T7-PA0920-$His_6$ was incubated with different radioactively labeled amino acids. The resulting lipids were extracted, subjected to 2D-TLC and autoradiography analysis. The following amino acids were added: *A*, 2.8 mM [U-$^{14}$C]-L-alanine. *B*, 2.8 mM [U-$^{14}$C]-L-lysine. *C*, 2.8 mM [U-$^{14}$C]-L-alanine and 2.8 mM [U-$^{14}$C]-L-lysine. The *black arrows* indicate the position of A-PG. The *ellipses* indicate the theoretical position of L-PG. Not converted radioactively labeled alanine is highlighted by *black arrow heads.*

Solely the addition of radioactively labeled alanine led to A-PG formation (Fig. 24, *A black arrow*). Addition of radioactively labeled lysine did not yield detectable L-PG amounts (Fig. 24, *B ellipse*). The addition of both, isotopically labeled alanine and lysine led to formation of significant amounts of A-PG whereas no L-PG synthesis was detectable (Fig. 24, *C*).

These experiments clearly indicate that A-PGS catalysis is highly specific for the biosynthesis of A-PG.

Recently, the *E. faecium* aa-PGS revealed a relaxed specificity for lysine, arginine and alanine under *in vitro* conditions. The orthologous *B. subtilis* protein showed a

specificity for lysine, in parallel with a relaxed specificity allowing for A-PG synthesis (Roy and Ibba, 2009). Interestingly, also a single strict specificity was determined for *A. tumefaciens* aa-PGS for lysine and for *C. perfringens* MprF1 for alanine (Roy and Ibba, 2009).

### 3.7.2 The tRNA identity of *P. aeruginosa* A-PGS

tRNA identity elements allow for the discrimination of tRNAs by aminoacyl-tRNA synthetases. The modification of PG catalyzed by the A-PGS was shown to be tRNA-dependent (chapter 3.2.1 and 3.6.2). Thus, the Ala-tRNA$^{Ala}$ has to be precisely identified by the A-PGS. To analyze the tRNA identity of *P. aeruginosa* A-PGS an *in vitro* activity assay was performed. For this approach tRNA$^{Ala}$ with posttranscriptional base modifications and unmodified tRNA$^{Ala}$ were investigated. Furthermore, several microhelices mimicking the minimal part of the tRNA$^{Ala}$ and tRNA$^{His}$ acceptor stem were used. tRNAs and tRNA microhelices were aminoacylated by their cognate aminoacyl-tRNA synthetase and tested as substrate for A-PG synthesis.
Therefore, tRNA$^{Ala}$ from *P. aeruginosa* was, *inter alia*, synthesized by *in vitro* transcription using purified T7 RNA polymerase. Furthermore, AlaRS from *P. aeruginosa* and *E. coli* and the histidyl-tRNA synthetase (HisRS) from *E. coli* were purified.

#### 3.7.2.1 Synthesis and Purification of two *P. aeruginosa* tRNA$^{Ala}$ Isoacceptors

The two isoacceptor tRNA$^{Ala}$ genes (PA4280.3, PA3133.2) from *P. aeruginosa* were cloned into the pUC18 and pUC119 vector system under control of the T7 promoter. *In vitro* transcription of these constructs after digestion with *Bst*NI yields unmodified tRNA transcripts with a 3'-CCA end (Sampson and Uhlenbeck, 1988). Therefore, T7 RNA polymerase was overproduced in *E. coli* BL21 (DE3)-RIL CodonPlus$^{TM}$ cells and purified *via* N-terminal His$_6$-tag. After *in vitro* transcription tRNA transcripts were purified *via* MonoQ chromatography according to Jahn *et al.* (1991) or alternatively *via* 12 % polyacrylamide gel electrophoresis in the presence of 8 M urea according to Pande

*et al.* (1991). Figure 25 summarizes the purification of the recombinantly produced T7 RNA polymerase and the anion exchange chromatography of $tRNA^{Ala1}$ from *P. aeruginosa.*

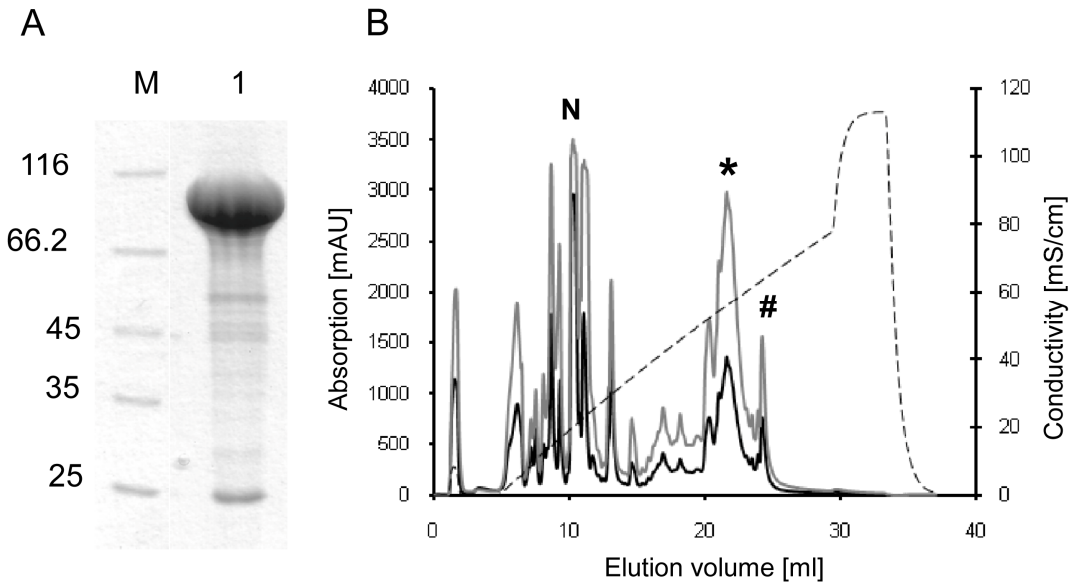



**Figure 25: SDS-PAGE analysis of purified T7 RNA polymerase (*A*) and purification of $tRNA^{Ala1}$ by anion exchange chromatography (*B*).**
*A*, The T7 RNA polymerase was recombinantly produced in *E. coli* BL21 (DE3)-RIL CodonPlus™ with an N-terminal $His_6$-tag, chromatographically purified, separated by 12 % SDS-PAGE, and visualized *via* Coomassie Brilliant Blue staining. *Lane M*, molecular mass marker, relative molecular masses (* 1'000) are indicated; *lane 1,* purified T7 RNA polymerase.
*B*, Elution diagram of $tRNA^{Ala1}$ purification by anion exchange chromatography: *in vitro* transcribed $tRNA^{Ala1}$ was loaded onto a MonoQ 5/50 GL column and eluted with a linear 20 ml gradient of sodium chloride from 0 to 750 mM at a flow rate of 0.5 ml $min^{-1}$ by monitoring absorbance (280 nm and 260 nm) and conductivity. The elution peak corresponding to $tRNA^{Ala1}$ is marked with an asterisk (*). The template-containing elution peak is highlighted by a number sign (#). Peaks "N" label nucleoside triphosphates.

Affinity chromatography *via* nickel-chelating sepharose resulted in highly enriched T7 RNA polymerase. The observed relative molecular mass for the T7 RNA polymerase was in good agreement with the calculated molecular mass of 98'000 (Fig. 25, *A*).

*In vitro* transcription of the two isoacceptor $tRNA^{Ala}$ genes from *P. aeruginosa* was performed using purified T7 RNA polymerase. Efficient *in vitro* transcription was indicated by the visible precipitation of $PP_i$. The subsequent purification of $tRNA^{Ala1}$ *via* anion exchange chromatography using a 20 ml gradient (0 - 750 mM NaCl) is shown in Figure 25 *B*. The respective nature of each individual peak was derived from MonoQ purification according to Jahn *et al.* (1991). Excess nucleoside triphosphates were

removed at a conductivity of 15 – 45 mS $cm^{-1}$ (peaks labeled N). $tRNA^{Ala1}$ eluted at a conductivity of 54 – 62 mS $cm^{-1}$ (peak labeled *) followed by the DNA template (peak labeled #). The template DNA was precipitated and re-used for a new round of *in vitro* transcription by T7 RNA polymerase.

tRNA containing fractions of MonoQ purification were ethanol precipitated and analyzed by polyacrylamide gel electrophoresis (Fig. 26).

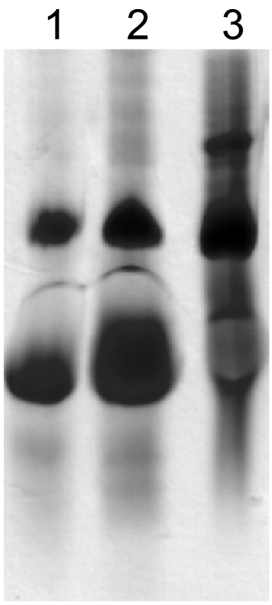



**Figure 26: PAGE analysis of purified $tRNA^{Ala}$ transcripts.**
Polyacrylamide gel analysis of purified *in vitro* transcribed $tRNA^{Ala1}$ and $tRNA^{Ala2}$. tRNA was separated using a 10 % polyacrylamide gel, and visualized *via* toluidine blue staining. *Lane 1*, *in vitro* transcribed $tRNA^{Ala1}$ from *P. aeruginosa*; *lane 2*, *in vitro* transcribed $tRNA^{Ala2}$ from *P. aeruginosa*; *lane 3*, tRNA Mix isolated from *E. coli* (Sigma Aldrich).

Purified *in vitro* transcribed $tRNA^{Ala1}$ (PA4280.3) and $tRNA^{Ala2}$ (PA3133.2) from *P. aeruginosa* (Fig. 26, *C lane 1, 2*) showed identical electrophoretic behavior compared to a tRNA Mix from *E. coli* (*lane 3*).

### 3.7.2.2 Purification of AlaRS and HisRS and Aminoacylation of tRNAs

Purified tRNAs and tRNA microhelices were aminoacylated by their cognate aminoacyl-tRNA synthetase. Therefore, the AlaRS and HisRS from *E. coli* were produced using the *E. coli* ASKA library clones JW2667 and JW2498 (Kitagawa *et al.*, 2005), respectively. *P. aeruginosa* AlaRS was recombinantly produced using *E. coli*

Rosetta (DE3) pLysS. Both AlaRS enzymes and HisRS were purified *via* $His_6$-tag affinity chromatography (Fig. 27).

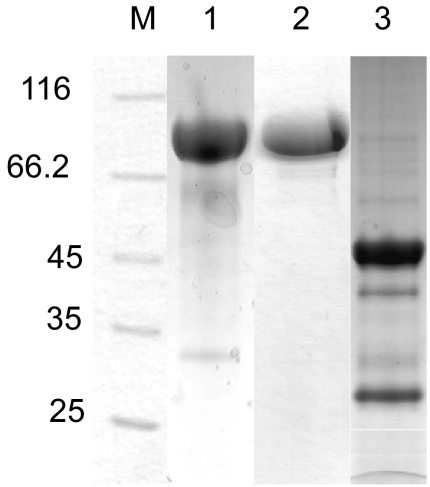



**Figure 27: SDS-PAGE analysis of purified AlaRS and HisRS.**
AlaRS and HisRS were recombinantly produced in *E. coli* with an N-terminal $His_6$-tag, chromatographically purified, separated by 12 % SDS-PAGE, and visualized *via* Coomassie Brilliant Blue staining. *Lane M*, molecular mass marker, relative molecular masses (* 1'000) are indicated; *lane 1,* purified AlaRS from *E. coli*; *lane 2*, purified AlaRS from *P. aeruginosa*; *lane 3*, purified HisRS from *E. coli*.

Affinity chromatography *via* nickel-chelating sepharose resulted in highly enriched to apparent pure AlaRS from *E. coli* (calculated molecular mass = 96'032 Da), AlaRS from *P. aeruginosa* (96'865 Da) and HisRS from *E. coli* (47'029 Da) (Fig. 27, *A lane 1-3*).

Aminoacylation activity of purified AlaRS from *E. coli* and *P. aeruginosa* and HisRS from *E. coli* was analyzed using radioactively labeled alanine or histidine. Based on the incorporation of radioactively labeled amino acids the radioactively labeled aminoacylated tRNA of an efficient aminoacylation reaction is precipitated by trichloroacetic acid and subsequently analyzed by scintillation counting (Fig. 28).

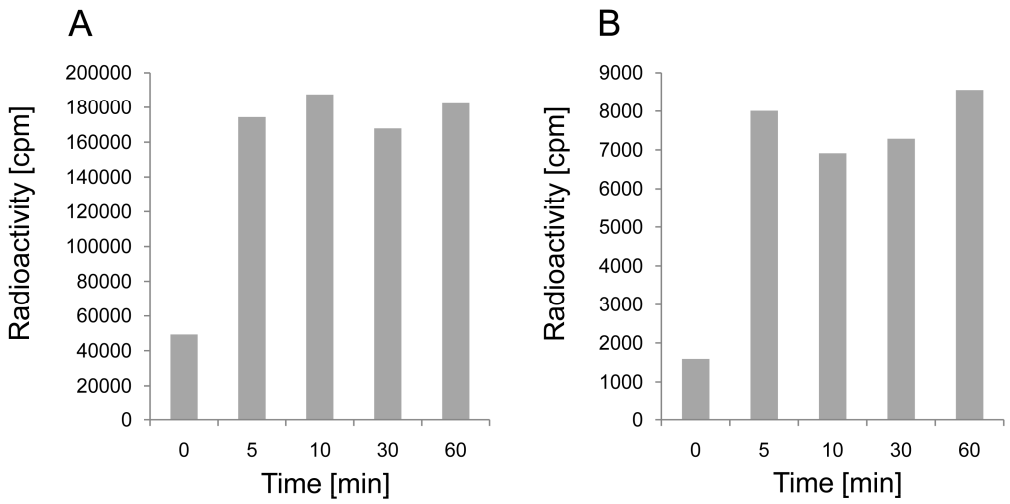



**Figure 28: Aminoacylation of $tRNA^{Ala1}$ (*A*) and microhelix F (*B*) by AlaRS and HisRS, respectively, from *E. coli*.**
*A*, AlaRS aminoacylation reaction was performed at RT according to Swairjo *et al.* (2004) using 4.75 µM of [2,3-$^{3}$H]-L-alanine, 10 µM of *in vitro* transcribed $tRNA^{Ala1}$ from *P. aeruginosa* and 1 µM of purified AlaRS from *E. coli*.
*B*, HisRS aminoacylation reaction was carried out at 37 °C according to Connolly *et al.* (2004) containing 21.7 µM of [U-$^{14}$C]-L-histidine, 10 µM of microhelix F (carrying $tRNA^{His}$ identity elements allowing for aminoacylation with histidine) and 1 µM of purified HisRS from *E. coli*.
At different time points samples were spotted onto Whatman filters (Whatman, Munich, Germany) and the tRNA was precipitated by incubation of the filters in 5 % (w/v) trichloroacetic acid for 10 min. Subsequently filters were subjected to 4 ml scintillation liquid and counts per minute were detected using a TriCarb 2900 TR scintillation counter.

Figure 28 exemplarily shows the aminoacylation of $tRNA^{Ala1}$ from *P. aeruginosa* by the *E. coli* AlaRS and microhelix F by the HisRS from *E. coli*, respectively. Microhelix F carries $tRNA^{His}$ identity elements allowing for aminoacylation with histidine. For both enzymes an increase of precipitated radioactivity was observed, indicating efficient aminoacylation activities (Fig. 28, *A and B*).

### 3.7.2.3 A-PGS Catalysis is Not Dependent on Posttranscriptional Modifications of tRNA

tRNA molecules are posttranscriptionally modified *in vivo*. $tRNA^{Ala}$ from *E. coli* contains the five base modifications 4-thiouridine, dihydrouracil, 7-methylguanosine, 5-methyluridine and pseudouridine (Fig. 29) (Ledoux *et al.*, 2009).

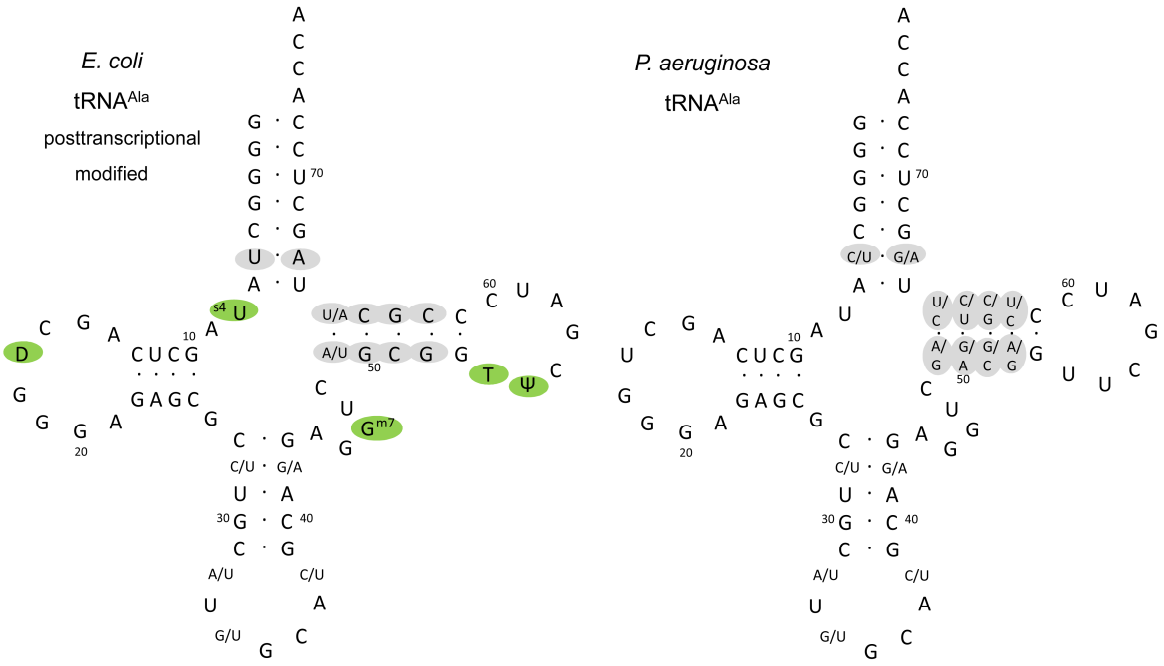



**Figure 29: Comparison of $tRNA^{Ala}$ from *E. coli* carrying posttranscriptional modifications and *in vitro* transcribed unmodified $tRNA^{Ala}$ from *P. aeruginosa*.**
Residues in smaller type are not conserved in the both isoacceptor $tRNA^{Ala}$ from *E. coli* and *P. aeruginosa*, respectively. Differences between $tRNA^{Ala}$ from *E. coli* and *P. aeruginosa* are highlighted in *grey*. Posttranscriptional base modifications of *E. coli* $tRNA^{Ala}$ are highlighted in *green*: $^{s4}U$ = 4-thiouridine, D = dihydrouracil, $G^{m7}$ = 7-methylguanosine, T = 5-methyluridine, Ψ = pseudouridine.

In order to investigate if posttranscriptional base modifications are required for A-PGS catalysis a coupled *in vitro* AlaRS/A-PGS activity assay was performed. Therefore, the assay mixture contained purified PG and recombinantly purified AlaRS in the presence of a commercially available mixture of posttranscriptional modified tRNAs from *E. coli*, or alternatively in the presence of purified unmodified $tRNA^{Ala1}$ or $tRNA^{Ala2}$ from *P. aeruginosa* which were produced by *in vitro* transcription (Sampson and Uhlenbeck, 1988) (Fig. 30). The lipids were extracted and $^{14}C$ labeled compounds were analyzed by autoradiography after 2D-TLC (Fig. 30).

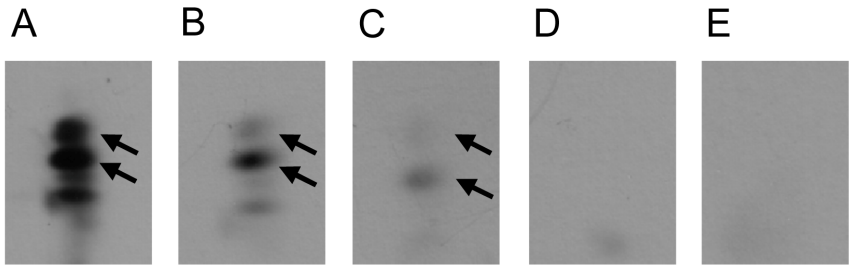



**Figure 30: *In vitro* formation of A-PG with posttranscriptional modified and unmodified tRNA$^{Ala}$.** *In vitro* assays with radioactively labeled [1-$^{14}$C]-L-alanine were performed as described in "Materials and Methods". After incubation of the assay for 1 h at 37 °C, lipids were extracted and subjected to 2D-TLC analysis. $^{14}$C-phospholipids were visualized by autoradiography. *A*, A-PG synthesis with *E. coli* posttranscriptional modified tRNA Mix as substrate. *B*, A-PG synthesis using *in vitro* transcribed tRNA$^{Ala1}$ as substrate. *C*, A-PG synthesis using *in vitro* transcribed tRNA$^{Ala2}$ as substrate. *D*, omitting GST-A-PGS$_{543-881}$ from the assay. *E*, After pre-treatment of the assay mixture with 100 µg ml$^{-1}$ RNase A for 25 min. A-PG is indicated by *arrows*.

In Figure 30, *A* the result of a typical *in vitro* A-PGS standard assay using a tRNA mixture with posttranscriptional base modifications from *E. coli* as substrate is shown. Clear A-PG synthesis was observed. This was also the case for experiments containing *in vitro* transcribed *P. aeruginosa* tRNA$^{Ala1}$ and tRNA$^{Ala2}$ which do not carry any posttranscriptional base modifications (Fig. 30, *B, C*). Control experiments in the absence of GST-A-PGS$_{543-881}$ or after RNase A pre-treatment did not result in any detectable A-PG synthesis (Fig. 30, *D, E*).

These experiments clearly indicate that the sequence variations of tRNA$^{Ala}$ from *E. coli* and *P. aeruginosa* (Fig. 29) do not abolish A-PG synthesis. Furthermore, it was concluded that the A-PGS tRNA substrate recognition is not dependent on posttranscriptional base modifications.

### 3.7.2.4 Substrate Specificity for the Acceptor Stem of Ala-tRNA$^{Ala}$

In *in vivo* and *in vitro* analyses of the A-PG formation by the A-PGS from *P. aeruginosa* it was clearly shown that the catalysis is highly specific for the transesterification of the alanyl-moiety from Ala-tRNA$^{Ala}$. Important experiments with relevance for the understanding of the substrate specificity of A-PGS have been already performed more than 40 years ago, when Gould and co-workers tested several modified Ala-tRNA derivatives as substrate for A-PGS from the Gram-positive bacterium *C. perfringens* (Gould *et al.*, 1968). All substrate analogs carrying modified amino acid

moieties (N-acetylalanyl-tRNA$^{Ala}$, lactyl-tRNA$^{Ala}$ and phenylalanyl-tRNA$^{Ala}$) did not sustain any A-PGS activity which clearly indicates that this part of the aminoacylated tRNA substrate is recognized with a high degree of specificity. Furthermore, it was shown, that the tRNA$^{Ala}$ part of the molecule is also relevant for substrate recognition, since Ala-tRNA$^{Cys}$ was not tolerated as an A-PGS substrate (Gould *et al.*, 1968). In the present study several microhelices (Fig. 31) were used to mimic the minimal part of the tRNA$^{Ala}$ acceptor stem to elucidate the main determinants of the *P. aeruginosa* A-PGS catalysis. Specific *P. aeruginosa* tRNA$^{Ala1}$ was synthesized by *in vitro* transcription and aminoacylated with radioactively labeled alanine. Purified aminoacylated tRNA$^{Ala1}$ or microhelices were subjected to the A-PGS *in vitro* assay. Subsequently, radioactively labeled phospholipids were extracted and analyzed by liquid scintillation analysis. The relative activity of A-PGS$_{543\text{-}881}$ for *in vitro* transcribed tRNA$^{Ala1}$ (Fig. 31) was set as 100 % and all other values were related to that.

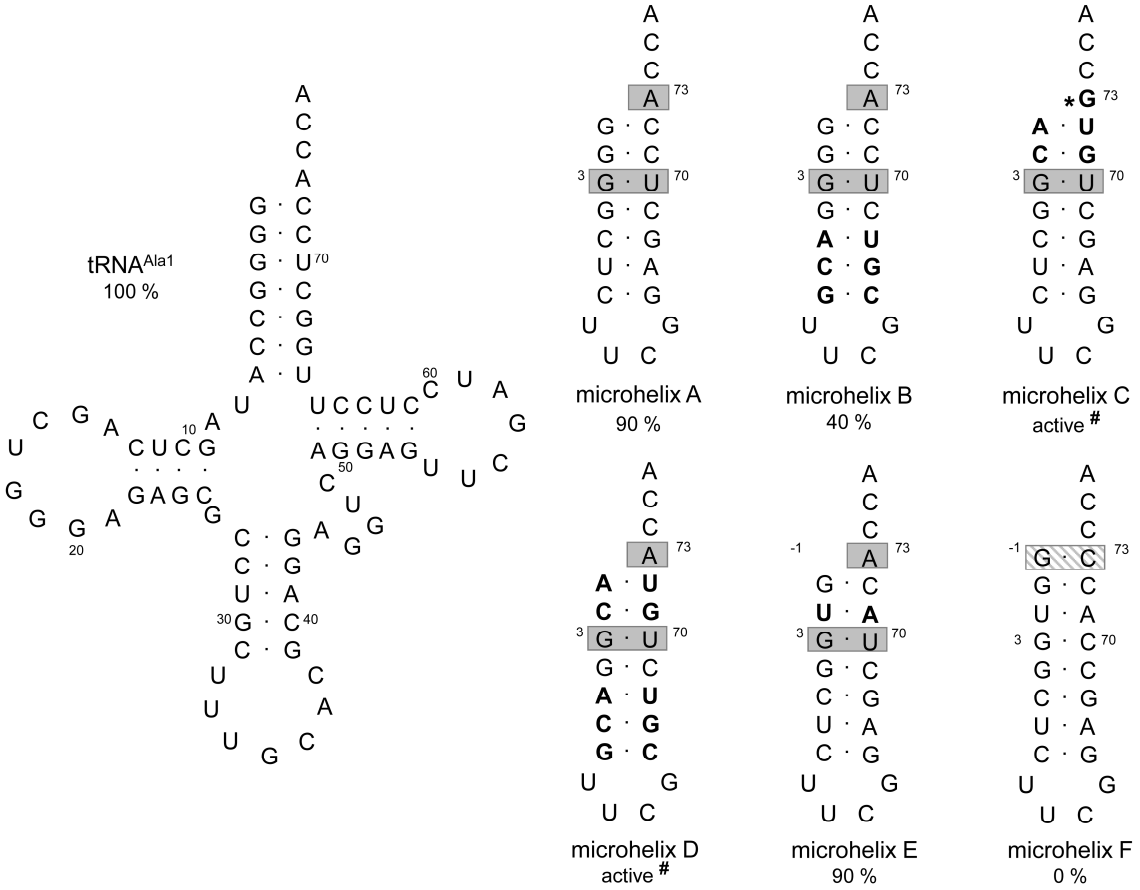



**Figure 31: Analysis of A-PGS substrate recognition by using derivatives of tRNA$^{Ala}$.**
Comparison of the tRNA sequences of the employed microhelices with the wild type tRNA$^{Ala1}$ sequence from *P. aeruginosa*. Bases that are essential determinants for *E. coli* AlaRS and for *E. coli* HisRS are highlighted with *grey boxes* and with a *striped box*, respectively. All bases that are modified when compared to microhelix A are highlighted in *bold font*. Numbering of microhelices is based on full-length *E. coli* tRNA$^{Ala}$. The relative activity of A-PGS$_{543-881}$ for *in vitro* transcribed tRNA$^{Ala1}$ was set as 100 %, obtained relative activities were related to that. *: Microhelix C - Efficient aminoacylation despite the mutated ´discriminator base` A73G has been shown earlier (Hou and Schimmel, 1988). #: Microhelix C and D were only inefficiently aminoacylated and therefore analyzed in a coupled AlaRS/A-PGS assay. Both microhelices served as active substrate in A-PG synthesis.

When microhelix A (Nagan *et al.*, 1999) was used as tRNA substrate analog a relative activity of 90 % was observed. This result clearly indicates that no D-loop, anticodon-loop, variable loop or TΨC-loop of the tRNA is required for A-PGS substrate recognition. The acceptor stem of this microhelix carries only five of the terminal base pairings (G1-C72, G2-C71, G3-U70, G4-C69, C5-G68) which can be found in the natural substrate. Accordingly all potential identity elements of this minimal substrate must be located on this part of the molecule. Therefore, microhelix B was analogously analyzed to characterize the potential role of base pair C5-G68. The enclosed mutation C5A-G68U resulted in a residual activity of only 40 %, which clearly indicates that this

position is highly relevant for A-PGS catalysis. Microhelix E also resulted in 90 % activity as observed for reference microhelix A, which clearly indicates that the U2-A71 base pair is not an important determinant for the A-PGS.

Bases G3, U70 and A73 are $tRNA^{Ala}$ identity elements of the AlaRS (Nagan *et al.*, 1999), these positions are essential for the efficient aminoacylation by AlaRS. Microhelix C or microhelix D carrying additional mutations at position G1-C72 (among other mutated positions) were only inefficiently aminoacylated in the employed assay. Therefore, a potential role as tRNA identity elements for A-PGS catalysis was analyzed in a coupled AlaRS/A-PGS assay. For both microhelices A-PG synthesis was observed. This type of analysis clearly indicated that mutation of position G1-C72 does not impede A-PG formation. These two experiments clearly rule out these two positions as tRNA identity elements for A-PGS catalysis.

Very early investigations with chemically modified ´alanyl-moieties` are indicative for a highly specific recognition of the alanyl part of the substrate (Gould *et al.*, 1968). To substantiate this idea for the A-PGS from *P. aeruginosa* microhelix F was aminoacylated with histidine. Comparison of microhelix F with microhelix E (which is an efficient substrate for A-PG synthesis) indicates only three variations of individual bases (G-1, C70, C73). This artificial substrate molecule did not result in any detectable A-PGS activity, suggesting that the alanyl-moiety is an important determinant for A-PGS substrate recognition. However, for this experiment an influence from the three base variations of microhelix F cannot be clearly ruled out.

From the results of the present study it was proposed that substrate recognition of the A-PGS enzyme only includes the acceptor stem of the tRNA substrate molecule. It was speculated that five of the terminal base pairings and especially the C5-G68 base pair are required to direct the alanyl-moiety of Ala-$tRNA^{Ala}$ into the active site of the A-PGS enzyme. tRNA identity elements for recognition of $tRNA^{Ala}$ by the AlaRS from *E. coli* compromise the discriminator base A73, the base pairings G2:C71, G3:U70 and G4:C69 of the acceptor stem in combination with base G20 of the D-loop. The anticodon loop is not needed for recognition (Hou and Schimmel, 1988; Francklyn and Schimmel, 1989; Tamura *et al.*, 1991). Figure 32 summarizes $tRNA^{Ala}$ identity elements for AlaRS and A-PGS.

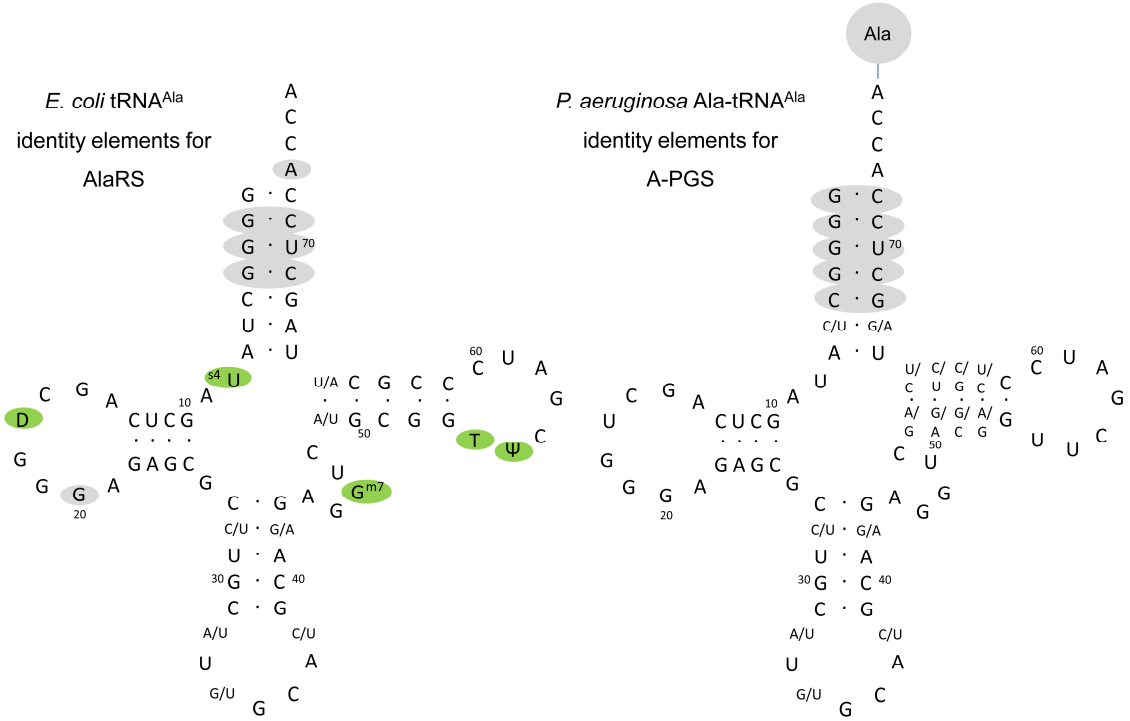



**Figure 32: Comparison of the tRNA$^{Ala}$ identity elements for *E. coli* AlaRS and *P. aeruginosa* A-PGS.**
tRNA Identity elements for recognition by AlaRS and A-PGS are highlighted in *grey*. A-PGS additionally recognizes the alanyl-moiety. Residues in smaller type are not conserved in the both isoacceptor tRNA$^{Ala}$ from *E. coli* and *P. aeruginosa*, respectively. Posttranscriptional base modifications of *E. coli* tRNA$^{Ala}$ are highlighted in *green*: $^{s4}$U = 4-thiouridine, D = dihydrouracil, G$^{m7}$ = 7-methylguanosine, T = 5-methyluridine, Ψ = pseudouridine.

Comparison of tRNA identity elements for both enzymes, the AlaRS and A-PGS, shows that major elements are located in the acceptor stem of the tRNA.

The glutamyl-tRNA reductase from *E. coli* catalyzes the initial step of tetrapyrrole biosynthesis by the NADPH-dependent reduction of tRNA-bound glutamate to glutamate-1-semialdehyde (Smith *et al.*, 1992). For this enzyme it was shown by analysis of 51 tRNA$^{Glu}$ variants, that the unique tertiary core structure of tRNA$^{Glu}$ plays an important role in substrate recognition. Besides this, neither the acceptor stem nor the anticodon stem are major tRNA identity elements for glutamyl-tRNA reductase (Randau *et al.*, 2004) in contrast to the glutamyl-tRNA synthetase (Sekine *et al.*, 1996). Furthermore, mischarged Gln-tRNA$^{Glu}$ was shown to serve as glutamyl-tRNA reductase substrate indicating a relaxed specificity for the amino acid residue (Lüer *et al.*, 2007).

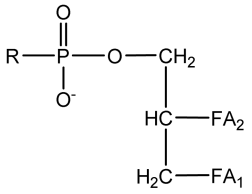
This might indicate that tRNA-dependent enzymes using the same tRNA as substrate recognize in some extent different structures and bases of tRNA.

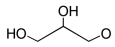
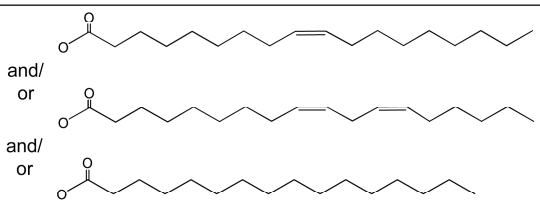
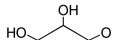
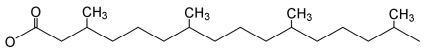
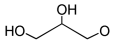
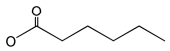
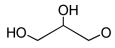
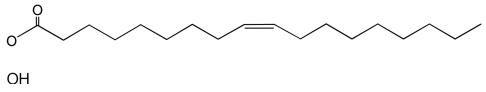
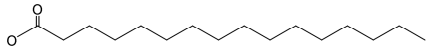
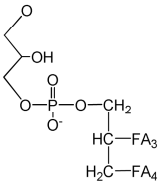
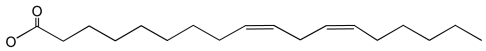
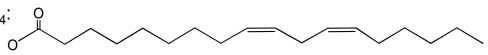
### 3.7.3 Specificity of PG Substrate Recognition

Phospholipids from *E. coli* are preferentially esterified with palmitic acid, hexadec-9-enoic acid and octadec-11-enoic acid, whereas phospholipids from *P. aeruginosa* are predominantly condensed with palmitic acid and methylene octadecanoic acid (Hancock and Meadow, 1969; Cronan and Rock, 1996). The employed *in vivo* activity assay might indicate that minor modifications of the fatty acid side chains of PG are tolerated by the A-PGS system. However, there are several investigations for phospholipid biosynthetic enzymes showing a high degree of fatty acid specificity (MacDonald *et al.*, 1988; Henneberry *et al.*, 2002). Besides the possibility to transplant active enzyme from several organisms into *E. coli*, there is no detailed experimental information about the specificity of aa-PGS towards the fatty acid residues on phosphatidylglycerol. Furthermore, only one publication deals with the head group specificity of L-PGS from *S. aureus* (Lennarz *et al.*, 1967).

To determine the specificity of A-PGS substrate recognition several derivatives of PG (Table 7) were analyzed in the *in vitro* activity assay. The most abundant fatty acids of the employed standard PG from egg yolk lecithin (Sigma-Aldrich) are oleic acid (18:1; (9*Z*)-octadec-9-enoic acid), linoleic acid (18:2; *cis, cis*-9,12-octadecadienoic acid) and palmitic acid (16:0; hexadecanoic acid) (Fredriksson *et al.*, 2006) (compare Table 7). In parallel control reactions in the absence of the phospholipid were employed.

**Table 7: Analysis of A-PGS substrate recognition by using derivatives of phosphatidylglycerol.**
Structures of the employed analogs of PG are indicated. R represents the polar head group of the phospholipid, $FA_1$ – $FA_4$ represents the individual fatty acid residues. For diphosphatidylglycerol (cardiolipin from bovine heart) (compound 5) the most abundant fatty acid residue is linoleic acid (Schlame and Otten, 1991). The relative activities of the individual compounds in the *in vitro* assay are indicated, standard deviations are ± 10 %.



| compound | phospholipid | R | $FA_1$ / $FA_2$ | activity |
|---|---|---|---|---|
| **PG** | PG from egg yolk lecithin |  |  | 100 % |
| **1** | 1,2-diphytanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) |  | 2 x  | 100 % |
| **2** | 1,2-dicaproyl-sn-glycero-3-phospho-(1'-rac-glycerol) |  | 2 x  | 20 % |
| **3** | 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-(1'-rac-glycerol) |  | and  | 20 % |
| **4** | 1,2-dipalmitoyl-sn-glycero-3-phospho(ethylene glycol) |  | 2 x  | 0 % |
| **5** | diphosphatidyl glycerol (cardiolipin from bovine heart) |  | 2 x  <br> $FA_3/FA_4$: 2 x  | 0 % |

To analyze the influence of the overall structure of the individual fatty acid, a PG molecule carrying two saturated C16 fatty acids, each modified by fourfold methylation (compound 1) was used. With this substrate no reduction of the specific activity was observed, which might indicate, that the integrity of the overall structure of the hydrophobic side chain (methylation or presence of double bonds) is not relevant for A-PGS substrate recognition. When a PG derivative containing two C6 fatty acids (instead of the usual C16 or C18 compounds) (compound 2) was investigated a residual activity of 20 % was determined which again demonstrates that the overall lipid fatty

acid residues are not recognized by the enzyme in detail. This conclusion was corroborated by using a PG derivative carrying solely one C18 fatty acid (compound 3). This truncated substrate molecule still resulted in a residual activity of 20 %. During A-PG catalysis the 2' hydroxy group or alternatively the 3' hydroxy group of the glycerophosphate moiety of PG nucleophilically attacks the carboxylate function of Ala-tRNA$^{Ala}$. To study the functional role of the polar head group of the phospholipid for A-PGS substrate recognition a truncated substrate carrying an ethylene glycol phosphate (compound 4) and a larger substrate carrying a diphosphatidylglycerol (additional with carrying four fatty acid residues) (compound 5) instead of the glycerol phosphate were also analyzed. These both substrate analogs did not result in any detectable A-PGS activity.

From these experiments it was concluded that the fatty acid residue of PG is not an important determinant for A-PGS substrate recognition, whereas the polar head group of A-PG is specific recognized.

## 3.8 Towards an Enzymatic Mechanism of A-PGS

The analysis of the enzymatic mechanism of A-PGS (or of orthologous enzymes) is of central importance for all subsequent efforts for the identification of efficient inhibitor molecules. For this purpose, the established *in vitro* activity assays in combination with classical chemical modification experiments and with an extended mutagenesis study was employed. Generally, there is the possibility of two different biosynthetic strategies for the formation of A-PG: A direct transesterification of the alanyl-moiety of Ala-tRNA$^{Ala}$ onto the 2´ or 3´ hydroxyl group of phosphatidylglycerol or alternatively a covalent catalysis *via* an enzyme localized alanyl ester intermediate. A direct transesterification mechanism might be metal ion assisted whereas formation of a covalent reaction intermediate requires the presence of a highly conserved nucleophilic residue (seryl, aspartyl, glutamyl, histidyl or cysteinyl).

A-PGS homolog proteins contain a highly conserved hydrophilic C-terminal domain sharing an overall of ~ 30 % sequence identity, and a less conserved N-terminal transmembrane domain (chapter 3.1). Furthermore, the truncated A-PGS$_{543\text{-}881}$ variant

from *P. aeruginosa* is sufficient for A-PG catalysis (chapter 3.6.1, 3.6.2), indicating that most probably the active site is located in this hydrophilic C-terminal domain.
It was suggested, that the essential amino acid residues for the recognition of the tRNA and the phospholipid substrate are conserved in all A-PGS homolog proteins. Therefore, highly conserved amino acid residues were exchanged by site directed mutagenesis, followed by a kinetic analysis of purified recombinant mutant A-PGS$_{543\text{-}881}$ proteins. In a second approach, purified recombinant wild type A-PGS$_{543\text{-}881}$ protein was modified by the reaction of different inhibitory compounds with specificity for specific amino acid residues or treated with chelating agents. Subsequently, these modified proteins were tested for activity.

### 3.8.1 A-PGS Catalysis is Not Dependent on a Highly Conserved Nucleophilic Residue

Presence of an activated nucleophilic side chain is an important feature of enzymes performing covalent catalysis (Scheiner and Lipscomb, 1975; Moser *et al.*, 1999; Perozich *et al.*, 1999; Iqbal *et al.*, 2009). In many cases compounds like phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DIPFP) (inhibiting serine residues) or iodacetamide (cysteine residues) allow for the direct chemical modification of such active site nucleophiles resulting in the covalent inactivation of the enzyme. When the A-PGS$_{543\text{-}881}$ from *P. aeruginosa* was treated with these three chemical modification reagents at concentrations up to 10 mM (iodacetamide) or up to 1 mM (PMSF and DIPFP) no significant loss of A-PGS activity was observed (Table 8).

**Table 8: Enzymatic activity of A-PGS$_{543-881}$ after chemical modification under *in vitro* conditions.** A-PGS$_{543-881}$ was purified in the absence of reducing agents and subsequently modified with phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate and iodacetamide, respectively. After an extensive dialysis step proteins were subjected to the standard *in vitro* assay using *E. coli* extracts as described in "Materials and Methods". The relative enzymatic activity of the corresponding unmodified A-PGS$_{543-881}$ was set as 100 %, and all other values were related to this value. Assays were performed in duplicate; standard deviations are ± 10 %.

| modifying agent | concentration [mM] | relative enzymatic activity [%] |
|---|---|---|
| phenylmethylsulfonyl fluoride (PMSF) | 0.1 | 100 |
| | 1 | 100 |
| diisopropyl fluorophosphate (DIPFP) | 0.1 | 100 |
| | 1 | 100 |
| iodacetamide | 1 | 100 |
| | 10 | 90 |

To substantiate these initial observations highly conserved amino acid residues with potential relevance for the catalytic mechanism of A-PGS were identified in an amino acid sequence alignment composed of A-PGS and L-PGS proteins from various organisms (Fig. 33).

```
Lm              HLWLVGFVGVFIAVVSLVIIYIYLSTTKEKLGSPFEAVKVREHLAKWG-GNEVSHTMFLR 546
Li              HLWLVGFVGVFIAVVSLVIIYIYLSTTKEKLGSPFEAVKVREHLAKWG-GNEVSHTMFLR 546
Cp2             KFGFIAFALVTVIYVAIYFLNIRRKIPVKTFDQCSEYIEK--IIEEYK-GDSLTHLVFLK 539
Sa              VLRYYFWLTILIIAIIIGMIAWLFDYQFSKVRISSKIEDCEEIINQYG-GNYLSHLIYSG 545
Sx              ILRYYFWITILLVAIIVGVIVWWFEYRYRSSNSRDNIATCESIIDKYN-GNYLSHLMYSG 545
Bc              VKRSALAAAFFVPTFLLIGSLIANRYRNEFPGQPANDKRLQNFLDEHG-GNVLSHLGFLG 563
Ba              VKRSALAAAFFVPTFLLIGSLIANRYRNEFPGQPANDKRLQNFLDEHG-GNVLSHLGFLG 563
Bs              ITHATIMAIIIVPLFFLIFTVVYHK-RTKPIGEKADPERLAAFLNEKG-GNALSHLGFLG 556
Cp1             YLRIALFTYISFIIFVIIWYLTMPKIEDDERYMDADLEKVSKFFKEIDYGTIFSHLVYLK 265
Pa              ALRAALGSCLLLLALALGWLLRAAPPAIREPN-AEELQRAARIIRHSD--QPDGGLALTG 578
                          . .  . :                  .       : .

Lm              DKLLFWAAEGEVLFSYRIIADKMVIMGEPTGNMDKMEAAIEEVMMNADRFGYRPVFYEVR 606
Li              DKLLFWAANGEVLFSYRIIADKMVIMGEPTGNMEKMEDAIEEVMTNADRFGYRPVFYEVR 606
Cp2             DKYIYLNEDKDLFIQYEVYGDKLFVLGNPVGNNENLFREIEKFCEYADNYGYTPVFYQVN 599
Sa              DKQFFTNENKTAFLMYRYKASSLVVLGDPLGDENAFDELLEAFYNYAEYLGYDVIFYQVT 605
Sx              DKKFFINDNKDAFVMYRYHNNTYIILGDPIGNSESFYSLLEAFYKEAEYLGYDIIFYQVT 605
Bc              DKQFFFSSDGKALLLFSITGKRLVVLGDPIGDPSSYRTVLQEFLAEADRFGYICVFYQIE 623
Ba              DKQFFFSSDGKALLLFSITGKRLVVLGDPIGDPSSYRTVLQEFLAEADRFGYICVFYQIE 623
Bs              DKRFYFSSDGNALLLFGKIARRLVVLGDPSGQRESFPLVLEEFLNEAHQKGFSVLFYQIE 616
Cp1             DKKVFWANEGESLIMYSKYKDKIIVLGDPIATKENLYSCIEEFQAFTNLYGYDVVFYEIE 325
Pa              DKALLFHESDDAFLMYARRGRSMIALYDPIGPAMQRAELIWQFRDLCDLHHARPVFYQVR 638
                **  .    .   :. :       . : :* .        :   .    .      :**::
                 D579 -> D579A, D579N

Lm              GTMIPYLHDHGFDFIKLGEEGFVDVQNFTMS--GKKKKGERALMNKLEREGYTFEIIEPP 664
Li              GTMIPYLHDHGFDFIKLGEEGFVDVQNFTMS--GKKKKGERALMNKLDREGYTFEIIQPP 664
Cp2             EEMISYLHSNGYDFMKIGEEAKVDVKEFKVV--GNKMKSLKTSRSKVTKEGYTFHMVEPP 657
Sa              DQHMPLYHNFGNQFFKLGEEAIIDLTQFSTS--GKKRRGFRATLNKFDELNISFEIIEPP 663
Sx              DKYMSLYHSFGNQFFKLGEEAVINLTSFTTS--GKKKRGLRATLNKLDDLGYSFEVLEPP 663
Bc              SKWMSLYHDFGYNFFKLGEEAVVDLNTFTIT--GKKRAGMRATFNRFEREGYTFSIHQPP 681
Ba              SKWMSLYHDFGYNFFKLGEEAVVDLNTFTIT--GKKRAGMRATFNRFEREGYTFSIHQPP 681
Bs              REDMALYHDFGYNFFKLGEEAYVDLNTFTLT--GKKKAGLRAINNRFEREEYTFHVDHPP 674
Cp1             EKNFSTYHDAGYYFFKLGEEARIDLEEFNLI--GSKKSAFRNTLRRVEREGYNFSIIEPP 383
Pa              AENLPFYMDIGLTALKLGEEARVDLLRFDLENKGKEMKDLRYTWNRGQRDGLALEFHEP- 697
                   :.   . *   :*:***. ::: *     *.:    :    :       : . .*
                                 E657 -> E657D, E657Q
                                  E658 -> E658D, E658Q
```

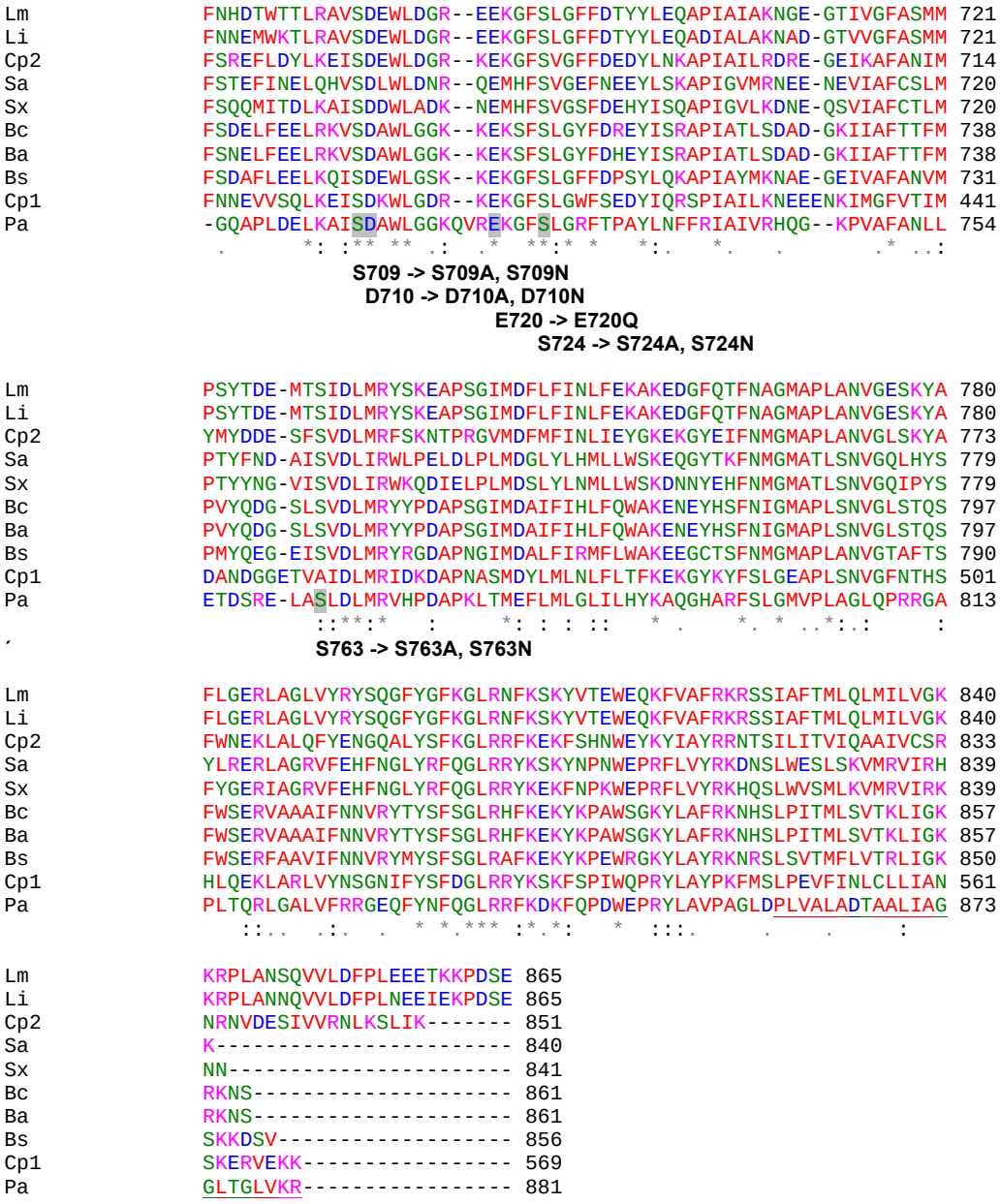



**Figure 33: Sequence alignment of the C-terminal domain of A-PGS homolog proteins.**
Homologous A-PGS proteins from *L. monocytogenes* (Lm), *L. innocua* (Li), *C. perfringens* MprF2 (Cp2), *S. aureus* (Sa), *S. xylosus* (Sx), *Bacillus cereus* (Bc), *B. anthracis* (Ba), *B. subtilis* (Bs), *C. perfringens* MprF1 (Cp1) and *P. aeruginosa* (Pa) were assembled with Clustal W2 (Larkin *et al.*, 2007). Identical residues are indicated by an *asterisk*, conserved substitutions by a *colon*, and semiconserved substitutions by a *period*. The first amino acid of the C-terminal hydrophilic A-PGS$_{543\text{-}881}$ variant is highlighted in *black*. The proposed C-terminal helix is *underlined*. Amino acid positions that have been mutagenized are highlighted in *grey*, point mutations that have been analyzed in the present study are indicated. The complete sequence alignment can be found in the appendix.

An overall of 8 amino acid residues which potentially might function as an active site nucleophile (D579, D710, E657, E658, E720, S709, S724, S763; *P. aeruginosa numbering*) were found 100 % conserved in all available A-PGS or L-PGS sequences of

the database. However, the employed sequence alignment did not reveal a highly conserved cysteinyl or histidyl residue. Therefore, involvement of a possible thiol ester intermediate was ruled out. To provide good insight into the functional role of the identified residues, each of these amino acid positions was mutagenized into a residue with a comparable size (conservative exchange) or alternatively into a residue with differing size or charge characteristics. The initial characterization of all mutant enzymes was performed in an *E. coli in vivo* activity assay analogously as described in a recent publication (Ernst *et al.*, 2009). This type of analysis only revealed mutant proteins showing activities similar to the wild type enzyme (E657Q, E658D, E658Q, E720Q, D710A, D710N, S709A, S709N, S763A, and S763N), mutants with decreased activities (D579A, D579N, E657D, and S724A) and with no detectable A-PGS activity (S724N) (Table 9). However a precise quantification of the relative A-PGS activity was hampered due to the accumulation of comparable amounts of A-PG in the bacterial membrane of *E. coli* despite greatly varying enzymatic activities (time course 3 h).

Though, the newly established *in vitro* assay using *E. coli* extracts allowed for the accurate determination of the specific activity of mutant proteins under initial-rate conditions. Therefore, lipid samples of the *in vitro* assay using *E. coli* extracts taken at various time points were analyzed by scintillation counting. All activities indicated in Table 9 are averaged on the basis of three independent experiments.

**Table 9: Overview of the relative activity of A-PGS$_{543-881}$ mutant proteins.**
Standard *in vivo* assays and *in vitro* assays using *E. coli* extracts were performed as described in "Materials and Methods". The specific activity observed for wild type A-PGS$_{543-881}$ was set as 100 % and all other values of mutant proteins were related to this value. A-PG formation in the *in vivo* assay was classified as: +++, activity comparable to wild type enzyme; +, decreased activity when compared to wild type enzyme; -, no detectable A-PGS activity.

| mutation | *in vivo* activity | relative enzymatic activity ± standard deviation [%] |
|---|---|---|
| Wild type A-PGS$_{543-881}$ | + + + | 100 ± 0 |
| D579A | + | 3 ± 1 |
| D579N | + | 5 ± 2 |
| E657D | + | 4 ± 1 |
| E657Q | + + + | 21 ± 4 |
| E658D | + + + | 28 ± 7 |
| E658Q | + + + | 19 ± 3 |
| S709A | + + + | 5 ± 1 |
| S709N | + + + | 17 ± 3 |
| D710A | + + + | 26 ± 3 |
| D710N | + + + | 30 ± 3 |
| E720Q | + + + | 10 ± 2 |
| S724A | + | 7 ± 2 |
| S724N | - | 0 ± 0 |
| S763A | + + + | 23 ± 5 |
| S763N | + + + | 11 ± 1 |

Numerous mutagenesis studies for enzymes employing a transient covalent bond of the substrate with residues in the active site resulted in a complete loss of enzymatic activity when the active site nucleophile residue was mutagenized (Carter and Wells, 1988; Corey and Craik, 1992; Moser *et al.*, 1999). However, mutagenesis of residues serine 709 and 763 into asparagine residues resulted in residual A-PGS activities of 17 ± 3 % and 11 ± 1 % when compared to the wild type enzyme. Also when those two residues were altered into alanine, residual activities of 5 ± 1 % and 23 ± 5 % were observed which rules out a potential role as an active site nucleophile. In contrast to this, mutant protein S724N did not sustain any A-PG formation. But the analysis of the more conservative mutant S724A clearly indicated a residual activity of 7 ± 2 %. This observation is not compatible with the involvement of a S724 nucleophile in the active site of A-PGS.

The significantly reduced activities of the employed mutant proteins might indicate that residues S709, S724 and S763 play an important role in A-PGS substrate recognition. Steric effects (S724A, S724N, S763A, and S763N) and/or the reduced ability for the formation of salt bridges might be responsible for the resulting activities.

Mutation experiments for residues aspartate 579 and 710 revealed an important role of these residues for A-PGS catalysis as indicated by residual activities of 3 ± 1 % and 5 ± 2 % (D579A, D579N) or 26 ± 3 % and 30 ± 3 % (D710A, D710N). Furthermore mutation of residues glutamate 657, 658 and 720 resulted in systematically decreased A-PGS activities of 4 ± 1 % or 21 ± 4 % (E657D, E657Q), 28 ± 7 % or 19 ± 3 % (E658D, E658Q) and of 10 ± 2 % (E720Q). Obtained values for this set of mutant enzymes argue for the involvement of these glutamate and aspartate residues in A-PGS catalysis. Steric effects or the absence of polar interactions upon substrate binding might be responsible for the reduced activities.

Taking into account the results of the chemical modification experiments and the results of the mutagenesis study it was concluded that the *P. aeruginosa* A-PGS does not employ a covalent catalysis. Therefore, a direct transesterification mechanism is concluded.

### 3.8.2 A-PGS Catalysis is Not Dependent on Metal Ions

To further elucidate the principles of A-PGS catalysis involvement of metal ions was analyzed. For this purpose the potential inhibitory effect of the chelating agents ethylenediaminetetraacetic acid and 1,10-phenanthroline were analyzed (Table 10).

**Table 10: Enzymatic activity of A-PGS$_{543-881}$ after treatment with chelating agents under *in vitro* conditions.**
A-PGS$_{543-881}$ was purified in the absence of reducing agents and subjected to the standard *in vitro* assay using *E. coli* extracts or *in vitro* activity assay as described in "Materials and Methods". Ethylenediaminetetraacetic acid and 1,10-phenanthroline, respectively, were added directly to the assays. The relative enzymatic activity of the corresponding unmodified A-PGS$_{543-881}$ was set as 100 %, and all other values were related to this value. Assays were performed in duplicate, standard deviations are ± 10 %. n.t. = not tested.

| modifying / chelating agent | concentration [mM] | relative activity determined under *in vitro* conditions using *E. coli* extracts [%] | relative *in vitro* activity [%] |
|---|---|---|---|
| ethylenediaminetetraacetic acid (EDTA) | 3 | 100 | 100 |
| | 10 | 100 | 100 |
| | 20 | n.t. | 100 |
| 1,10-phenanthroline | 1 | 100 | n.t. |
| | 3 | n.t. | 100 |
| | 5 | n.t. | 75 |
| | 10 | 45 | 35 |
| | 20 | n.t. | 20 |

When the *in vitro* activity assay was performed in the presence of up to 20 mM EDTA or up to 5 mM 1,10-phenanthroline no significant inactivation was observed. However, in the presence of 10 mM and 20 mM 1,10-phenanthroline a residual activity of approximately 35 % and 20 %, respectively, was determined. Since these experiments could not clearly rule out the involvement of a "tightly bound" metal ion the purified protein GST-A-$PGS_{543-881}$ was concentrated (10.5 mg $ml^{-1}$ - 161 µM) and subjected to inductively coupled plasma - mass spectrometry (ICP-MS) experiments (Table 11).

**Table 11: Determination of metal ion content by ICP-MS analysis.**
Purified GST-A-$PGS_{543-881}$ protein was concentrated and subjected to inductively coupled plasma - mass spectrometry (ICP-MS) experiments. In parallel the sole GST protein (4 mg $ml^{-1}$ - 154 µM) was analyzed to determine the background level of the employed purification strategy. Experiments were performed in duplicates.

| metal ion | concentration of metal ion in GST-A-$PGS_{543-881}$ [µM] | concentration of metal ion in GST [µM] |
|---|---|---|
| $Cu^{2+}$ | 0.3 | 0.2 |
| $Fe^{2+}$ | 4.3 | 1.6 |
| $Mg^{2+}$ | 18.8 | 1.2 |
| $Mn^{2+}$ | 0.4 | 0.02 |
| $Ni^{2+}$ | 0.2 | 0.2 |
| $Zn^{2+}$ | 0.9 | 0.2 |

This type of analysis did not reveal any ferrous, manganese, nickel, copper, or zinc ions above the background level of the employed buffer system (Table 11). The purified protein fraction only revealed substoichiometric amounts of magnesium (19 µM). In the context of the dramatically higher chelator concentrations of the employed inhibition experiments (10 mM - 20 mM) metal-dependent A-PGS catalysis was ruled out.

### 3.8.3 Chemical Modification of Lysine and Arginine Residues

Chemical modification experiments in the presence of citraconic anhydride (modification of lysine residues) and p-hydroxyphenylglyoxal (modification of arginine residues) at concentrations of up to 10 mM were performed to identify lysine and arginine residues which are involved in the catalytic mechanism of A-PG synthesis (Table 12).

**Table 12: Enzymatic activity of A-PGS$_{543-881}$ after treatment with chelating agents under *in vitro* and *in vitro* (using Ala-tRNA$^{Ala}$) conditions.**
A-PGS$_{543-881}$ was purified in the absence of reducing agents and subsequently modified with citraconic anhydride and p-hydroxyphenylglyoxal, respectively, subjected to the standard *in vitro* assay using *E. coli* extracts or *in vitro* assay as described in "Materials and Methods". After an extensive dialysis step proteins were subjected to the standard *in vitro* A-PGS$_{543-881}$assays. The relative enzymatic activity of the corresponding unmodified A-PGS$_{543-881}$ was set as 100 %, and all other values were related to this value. Assays were performed in duplicate, standard deviations are ± 10 %. n.t. = not tested.

| modifying agent | concentration [mM] | relative activity determined under *in vitro* conditions using *E. coli* extract [%] | relative *in vitro* activity [%] |
|---|---|---|---|
| citraconic anhydride | 2 | 100 | n.t. |
| | 10 | 100 | n.t. |
| p-hydroxyphenylglyoxal | 0.5 | 90 | n.t. |
| | 1 | 90 | 100 |
| | 5 | 60 | n.t. |
| | 10 | 60 | 100 |

Although, amino acid exchanges of K547, K621, R734A, and K806 of *S. aureus* MprF lead to completely abrogate L-PG synthesis under *in vivo* conditions (Ernst *et al.*, 2009) the results of chemical modification experiments of *P. aeruginosa* A-PGS do not indicate the critical involvement of lysine and arginine residues in A-PGS catalysis.

## 3.9 The Postulated C-terminal Helix of A-PGS is Essential for A-PG Synthesis

Bioinformatical analysis revealed a transmembrane segment located at the C-terminus of the A-PGS protein (Fig. 7). This putative transmembrane helix was elucidated for the involvement in A-PG catalysis. Based on theoretical analyses of the aa-PGS from *B. subtilis* and *C. perfringens* an additional C-terminal transmembrane helix was proposed (Roy and Ibba, 2009). Computational analysis of the overall domain architecture of *P. aeruginosa* A-PGS also revealed a potential transmembrane helix (amino acid residues 860 to 879) located at the C-terminus of the membrane protein (Fig. 7). To elucidate the functional role of this putative transmembrane segment of A-PGS two C-terminally truncated variant proteins were analyzed for activity: A-PGS$_{1-855N}$ and A-PGS$_{543-855N}$. For investigation of *in vivo* activity, A-PGS$_{1-855N}$ and A-PGS$_{543-855N}$ were recombinantly overproduced in *E. coli* TOP10 and BL21 (λDE3),

respectively. The extracted lipids were separated by 2D-TLC and visualized as outlined before (Fig. 34).

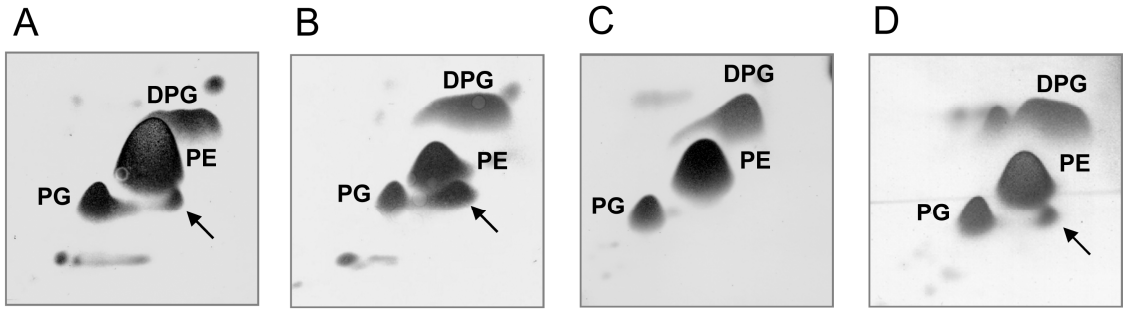



**Figure 34: Lipid composition of *E. coli* cells overproducing different A-PGS variants.**
*E. coli* BL21 (λDE3) or TOP10 harboring the respective plasmid were cultivated at 37 °C and 200 rpm to an $OD_{578}$ of 0.5. Recombinant gene expression in BL21 (λDE3) and TOP10 was induced by addition of 50 µM IPTG and 0.02 (w/v) % L-arabinose, respectively. Cells were further incubated for 3 h or 4 h and harvested. The lipids were extracted, separated by 2D-TLC and visualized by spraying with 5 % molybdatophosphoric acid. Lipid composition of: *A,* A-$PGS_{543-855N}$ overproducing BL21 (λDE3). *B*, A-$PGS_{543-881}$ overproducing BL21 (λDE3). *C*, A-$PGS_{1-855N}$ overproducing TOP10. *D*, A-PGS overproducing TOP10. A-PG is indicated by an *arrow*. PE = phosphatidylethanolamine, PG = phosphatidylglycerol, DPG = diphosphatidylglycerol.

In the employed *in vivo* activity assay the A-$PGS_{1-855N}$ mutant did not sustain any detectable A-PGS activity (Fig. 34, *C*). This was also the case when the purified mutant protein A-$PGS_{543-855N}$ was analyzed in the *in vitro* assay using *E. coli* extracts (data not shown). However, under *in vivo* conditions A-PG formation for the A-$PGS_{543-855N}$ mutant was observed (Fig. 34, *A*). The amount of A-PG was significantly decreased in comparison to A-$PGS_{543-881}$ variant (Fig. 34, *B*) indicating a critical involvement of the C-terminal transmembrane domain into catalysis.

From these results it was concluded that amino acid residues 856 - 881 play a crucial role for the activity of *P. aeruginosa* A-PGS. Due to the hydrophobic nature of this protein segment a potential involvement in the recognition of the lipid substrate might be speculated.

## 3.10 Cellular Localization of A-PGS and A-PG in *P. aeruginosa*

### 3.10.1 Biochemical Approach for the Subcellular Localization of A-PGS

Bioinformatic analysis identified A-PGS as a transmembrane protein containing 14 transmembrane helices located in the cytoplasmic membrane. To experimentally elucidate cellular localization of A-PGS, membrane fractions were prepared from *E. coli* strain TOP10 carrying pBAD-His-A/PA0920. To determine the precise localization of A-PGS in *E. coli*, a separation of the cytoplasmic and outer membrane fraction was performed using a well-established procedure (Yamato *et al.*, 1975). The concluded localization of A-PGS in the cytoplasmic membrane was further elucidated using a second independent approach. This experiment made use of the ability to selectively solubilize membrane proteins of the cytoplasmic membrane using sarcosyl as a detergent (Eitel and Dersch, 2002) (Fig. 35).

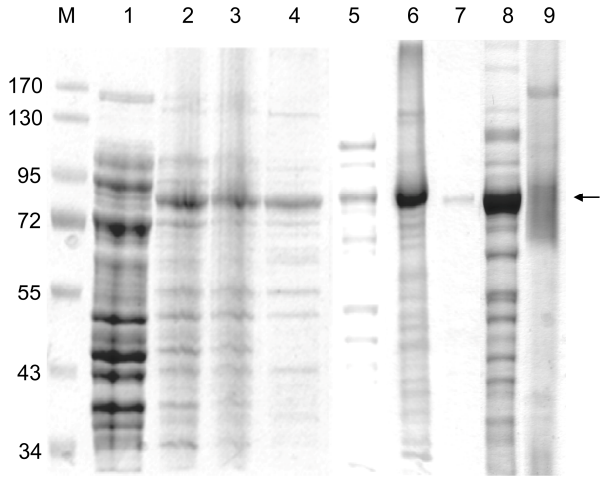



**Figure 35: SDS-PAGE analysis of membrane fraction preparation and separation of cytoplasmic and outer membrane.**
*E. coli* TOP10 harboring pBAD-His-A/PA0920 was cultivated as described in "Materials and Methods". The membrane fraction was purified by discontinuous sucrose gradient centrifugation. The cytoplasmic and outer membrane was separated by two independent approaches according to Yamato *et al*. (1975) and Eitel & Dersch (2002). Proteins were separated by 9 % SDS-PAGE, and visualized *via* Coomassie Brilliant Blue staining. *Lane M*, molecular size marker, relative molecular masses as indicated (*1'000). *Lane 1*, cytosolic fraction after cell disruption. *Lane 2*, insoluble fraction after cell disruption. *Lane 3*, membrane fraction obtained by discontinuous sucrose gradient centrifugation. *Lane 4*, Triton X-100-solubilized membrane proteins. *Lane 5*, purified A-PGS after affinity chromatography using chelating sepharose. *Lane 6*, cytoplasmic membrane fraction prepared as described in Yamato *et al*. (1975). *Lane 7*, outer membrane fraction prepared as described in Yamato *et al*. (1975). *Lane 8,* sarcosyl-solubilized cytoplasmic membrane proteins according to Eitel & Dersch (2002). *Lane 9*, outer membrane proteins after sarcosyl solubilization method according to Eitel & Dersch (2002). The position of A-PGS is indicated by an *arrow*. It is important to mention that A-PGS samples had to be incubated at 40 °C instead of the 95 °C with standard SDS-PAGE loading buffer to prevent the membrane protein from aggregation.

After cell disruption and centrifugation, A-PGS was detected in the insoluble and the membrane fraction (Fig. 35, *lane 2, 3*) but was completely absent from the cytosolic fraction (Fig. 35, *lane 1*). For purification of A-PGS the obtained membrane fraction was solubilized using Triton X-100 (Fig. 35, *lane 4*). Approximately 80 % of the overall A-PGS protein was found solubilized. Furthermore, in the solubilized fraction A-PGS appeared as the prominent protein band. To further purify A-PGS, a standard protocol for the purification of $His_6$-tagged proteins in the presence of 9 mM Triton X-100 was employed. A-PGS was successfully eluted in the presence of 500 mM imidazole (Fig. 35, *lane 5*).

The separation of the cytoplasmic and outer membrane fraction according to Yamato *et al.* (1975) revealed A-PGS as one of the most dominant proteins of the cytoplasmic membrane fraction (Fig. 35, *lane 6*), whereas A-PGS was almost completely absent in the outer membrane (Fig. 35, *lane 7*). Also, the selective solubilization of cytoplasmic membrane proteins using sarcosyl (Eitel and Dersch, 2002) determined the A-PGS protein as the most dominant protein of the solubilized cytoplasmic membrane fraction (Fig. 35, *lane 8*). Only trace amounts of A-PGS were detected in the outer membrane fraction (Fig. 35, *lane 9*).

Based on these results it was concluded that A-PGS is a cytoplasmic transmembrane protein.

Interestingly, the theoretical relative molecular mass of ~ 98'000 Da differs significantly from the experimental determined molecular mass of $His_6$-A-PGS (Fig. 35) and A-PGS-myc-$His_6$ (data not shown). It was supposed that this phenomenon results from the extended hydrophobic segments of the membrane protein as described for other membrane proteins (Kramer *et al.*, 2000).

### 3.10.2 Cellular Localization of A-PG

The biophysical separation of the cytoplasmic and the outer membrane was also used to analyze the localization of the reaction product of the A-PGS catalysis. Phospholipids of both membrane fractions were extracted and separated by 2D-TLC (Fig. 36).

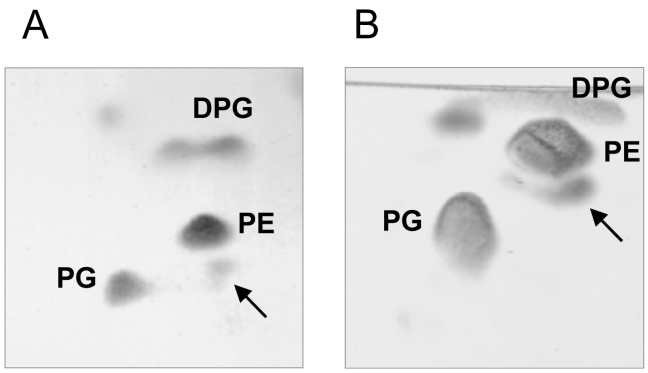



**Figure 36: 2D-TLC analysis of cytoplasmic and outer membrane phospholipids.**
Cytoplasmic and outer membrane fractions were prepared from *E. coli* strain TOP10 carrying pBAD-His-A/PA0920 according to Yamato *et al.* (1975). The lipids from these fractions were extracted, separated by 2D-TLC and visualized by spraying with 5 % molybdatophosphoric acid. *A*, Lipid composition of the cytoplasmic membrane fraction. *B*, Lipid composition of the outer membrane fraction. A-PG is indicated by *arrows*. PE = phosphatidylethanolamine, PG = phosphatidylglycerol, DPG = diphosphatidylglycerol.

The cytoplasmic and the outer membrane fraction of A-PGS overproducing *E. coli* cells contained almost identical amounts of A-PG (Fig. 36, *A, B*).

From these results it was concluded that the A-PGS-dependent biosynthesis is located in the cytoplasmic membrane whereas the resulting phospholipid A-PG is a constituent of the cytoplasmic and the outer bacterial membrane. The mechanism by which A-PG is transported from the proposed place of A-PG synthesis - the inner leaflet of the cytoplasmic membrane – to the outer leaflet of cytoplasmic membrane and into the outer membrane remains to be determined. Bacterial phospholipid translocation by flippases is only poorly understood. However, only recently a putative flippase activity for the N-terminal transmembrane domain of *S. aureus* MprF was proposed (Ernst *et al.*, 2009). In future experiments a possible flippase activity of the N-terminal domain of A-PGS from *P. aeruginosa* has to be investigated.

### 3.10.3 Microscopic Localization of the *P. aeruginosa* A-PGS

To investigate the location of *P. aeruginosa* A-PGS and especially its postulated C-terminal transmembrane helix (compare chapter 3.1) the subcellular localization of A-PGS and the C-terminus of A-PGS by immunogold labeling of ultrathin plastic-embedded cell sections was determined. For this purpose a myc epitope C-terminally fused to wild type A-PGS was immune electron microscopically detected, with a myc-specific antibody (Fig. 37).

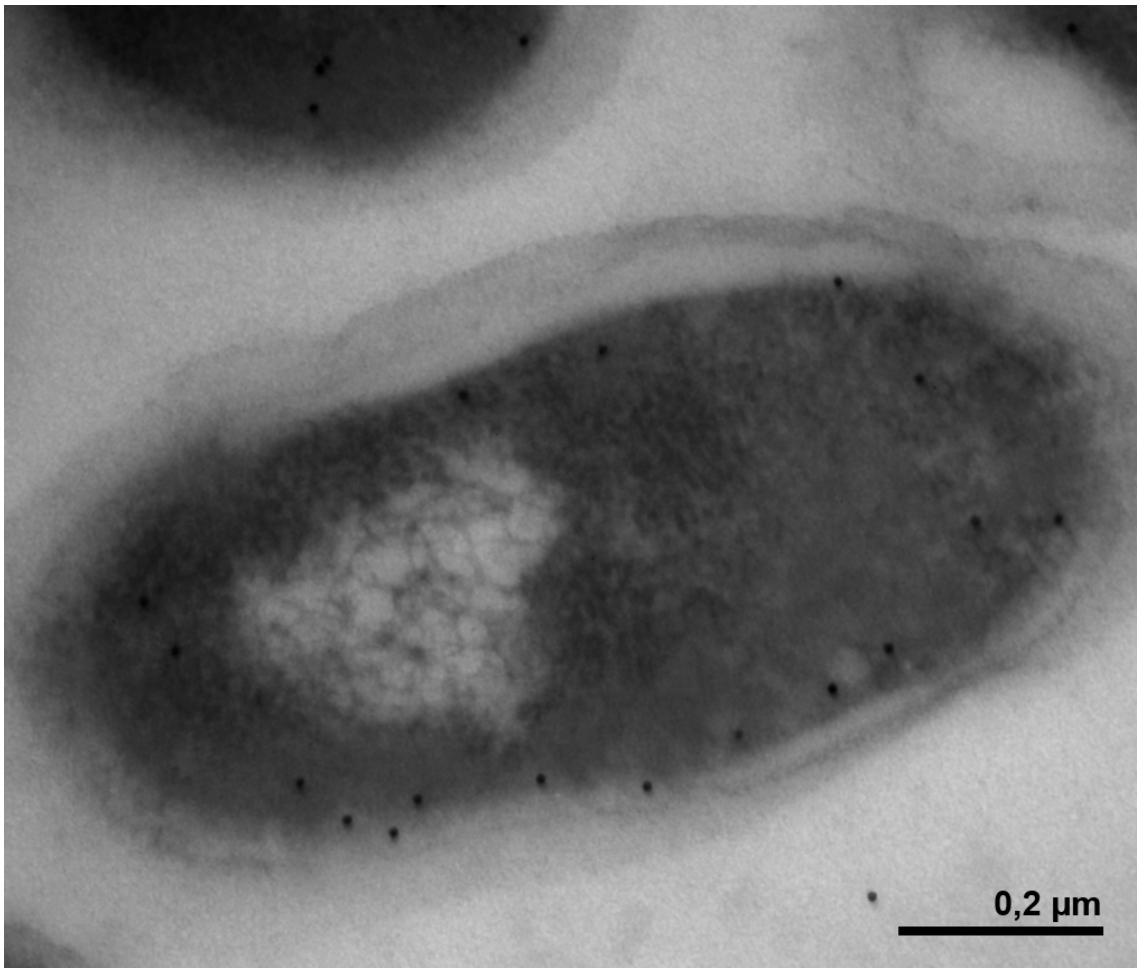



**Figure 37: Localization analysis of A-PGS and its C-terminus by immunogold labeling.**
*E. coli* TOP10 pBADmyc-His-A/PA0920 was cultivated as described in "Materials and Methods". The subcellular localization of a myc epitope C-terminally fused to the A-PGS was immune electron microscopically detected. Immunogold labeling was performed in the presence of a myc-specific antibody. Gold particles, indicative for the localization of the myc epitope, almost exclusively decorate the cytosolic part of the cytoplasmic membrane.

In Figure 37 gold particles, indicative for the localization of the myc epitope, almost exclusively decorate the cytosolic part of the inner membrane. This new methodology

revealed a homogeneous distribution of A-PGS in the cytoplasmic bacterial membrane. Besides this, the experiment confirms the results of previous biochemical analysis which indicated the localization of A-PGS in the cytoplasmic bacterial membrane (chapter 3.10.1). Furthermore, the localization of the myc epitope revealed a cytoplasmic orientation of the C-terminus of A-PGS. However, this observed cytoplasmic orientation is in disagreement with the computational prediction (Fig. 7). Based on these labeling experiments it was concluded that the postulated C-terminal helix is not a transmembrane helix that penetrates the bacterial membrane. This part of the molecule might be a hydrophobic protein segment which is part of the soluble catalytic domain. These results were also confirmed by localization analysis of constructs A-$PGS_{543\text{-}855N}$ and A-$PGS_{543\text{-}881}$. After the separation of the membrane fraction from the cytosolic fraction both protein variants were exclusively detected in the soluble protein fraction (data not shown). Besides this, gel permeation experiments (chapter 3.5.2) revealed a monomeric, monomodal protein fragment A-$PGS_{543\text{-}881}$ which is inconsistent with a highly hydrophobic transmembrane segment.

The C-terminal localization and the requirement of residues 856 - 881 for A-PGS activity might indicate that this C-terminal part of the molecule is directly involved in substrate recognition and/or catalysis. Furthermore, *P. aeruginosa* A-PGS does not possess a C-terminal transmembrane helix.

# 4 SUMMARY

Aminoacyl-phosphatidylglycerol synthases (aa-PGS) catalyze the tRNA-dependent modification of the phospholipid phosphatidylglycerol (PG) with alanine, lysine and arginine, respectively, resulting in a significant change of membrane biochemistry. However, the exact biological roles and catalytic mechanisms of these enzymes were poorly understood. Due to the contribution to antibiotic resistance and host adaptation this enzyme of the opportunistic pathogen *P. aeruginosa* is of special interest.

In this study, the alanyl-phosphatidylglycerol (A-PG) formation in Gram-negative *P. aeruginosa* PAO1 was investigated. Analysis of the membrane composition of the bacterium grown under acidic conditions (pH 5.3) revealed up to 6 % of A-PG of the total amount of phospholipids compared to almost no A-PG at neutral pH. In agreement, the corresponding PA0920 gene encoding alanyl-phosphatidylglycerol synthase (A-PGS) was found significantly upregulated under acidic conditions. Formation of A-PG was found responsible for the resistance of *P. aeruginosa* to the antibiotic cefsulodin, the heavy metal ion $Cr^{3+}$, the osmolyte sodium lactate, and the cationic peptide protamine sulphate. Clearly, alanylation of the membrane serves multiple adaptation reactions to environmental stresses.

In a second part of this study a recombinant minimal *P. aeruginosa* A-PGS catalytic fragment was produced and employed as a versatile tool for the elucidation of the enzymatic mechanism *in vitro*. This robust test system clearly reflected the *in vivo* properties of A-PGS catalysis. Based on a site-directed mutagenesis study with 15 mutant A-PGS proteins in combination with chemical enzyme modification experiments a direct transesterification mechanism for A-PGS catalysis was proposed. Determinants of the substrates were elucidated. The polar head group of PG is specifically recognized, whereas the fatty acid residue of PG is not an important determinant for A-PGS substrate recognition. The tRNA substrate recognition of the A-PGS enzyme does not include posttranscriptional base modifications. Besides this, only the acceptor stem of the overall structure of the tRNA is recognized. Based on A-PGS activity assays with 6 different tRNA microhelices it was speculated that five of

the terminal base pairings and especially the C5-G68 base pair are required to direct the alanyl-moiety of Ala-tRNA$^{Ala}$ into the active site of the enzyme.

Electron microscopy in combination with immunogold-labeling revealed a homogeneous distribution of *P. aeruginosa* A-PGS in the cytoplasmic bacterial membrane. Finally, the cytoplasmic location of the C-terminus of the membrane protein which is relevant for A-PGS activity was determined.

Obtained results provided first significant insights into the enzymatic mechanism of A-PG formation which provide a solid basis for development of A-PGS inhibitory molecules in the future.

# 5 OUTLOOK

As outlined above, a detailed biochemical characterization of A-PGS from *P. aeruginosa* was performed in this study. The following questions have to be addressed in future experiments:

- determination of the three-dimensional structure of A-PGS
- identification of amino acid determinants for the specificity of aa-PGS enzymes
- complementation of the *P. aeruginosa* ΔPA0920 deletion mutant strain with L-PGS to characterize the functional role of A-PG *versus* L-PG formation

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# 7 APPENDIX

## Sequence alignment of orthologous A-PGS proteins

Homologous aa-PGS proteins from *Listeria monocytogenes* (Lm), *Listeria innocua* (Li), *Clostridium perfringens* MprF2 (Cp2), *Staphylococcus aureus* (Sa), *Staphylococcus xylosus* (Sx), *Bacillus cereus* (Bc), *Bacillus anthracis* (Ba), *Bacillus subtilis* (Bs), *Clostridium perfringens* MprF1 (Cp1) and *Pseudomonas aeruginosa* (Pa). Identical residues are indicated by an *asterisk*, conserved substitutions by a *colon*, and semiconserved substitutions by a *period*. The first amino acid of the soluble A-PGS$_{543\text{-}881}$ variant is highlighted in *black*. The proposed C-terminal helix is *underlined*. Amino acid positions that have been mutagenized are highlighted in *grey*, point mutations that have been analyzed in the present study are indicated.

## CLUSTAL 2.0.12 multiple sequence alignment

```
Lm              ----------------MKEKLMQAYAWFQKNSTVVKIVFITFVMAFVIFEIINIATGID 43
Li              ----------------MKEKLMQAYAWFQRNSMIVKIVFITFVMGFVIFEIVNIATGID 43
Cp2             --------------------VKLNIKISDKLKLFFKIAFVSLVILFIVREFTSVFKNFN 39
Sa              ----------------------MNQEVKNKIFSILKITFATALFIFVAITLYRELSGIN 37
Sx              ----------------------MTKELRSKLFTILKIAFALTLFTIVAITLYKELSHIN 37
Bc              ------------------------MSFSWKRFLQIGKIIFPFVVLTIVFFQAKKELAGIS 36
Ba              ------------------------MSFSWKRFLQIGKIIFPFVVLTIVFFQAKKELAGIS 36
Bs              -------------------------MLIKKNALSILKIVFPIAVLLFVIYQSKKELTNLS 35
Cp1             ------------------------------------------------------------
Pa              MRTDAPVPEHPAPPSSPASPQRIRLIDRITAYRQPIGLVFTLLLFGLALVACYHLLREID 60

Lm              YPSLKENLTSQSPEQIFIMFIVGLIAVTPMLLYDYVIVKLLPGKFSPSHVIASGWITNTF 103
Li              YPSLKANITSQSPEQIFIMFIVGLIAVTPMLLYDYVIVKLLPGKFSPIHVVASGWITNTF 103
Cp2             SEYFFMYRNKLDFLNLLIIAALGVISYIPLSFYDFILKRKVRIRLKNRKLYKYSWIASSI 99
Sa              FKDTLVEFSKINRMSLVLLFIGGGASLVILSMYDVILSRALKMDISLGKVLRVSYIINAL 97
Sx              LKDAIKSFSKINRFWLVALFLSGGASIIVLSIYDVILAKTLKLKIGLAKTIRIGYIVNAL 97
Bc              FLEAINTIKNIPTGGVFLAITLGAFAVSTMFFYDYVMLRYLKADIPVQKIFRISWIANTL 96
Ba              FLEAINTIKNIPTGGVFLAITLGAFAVSTMFFYDYVMLRYLKADIPVQKIFRISWIANTL 96
Bs              FKRTLMVINGLERTDLFMLVLIGLLAVAAMSLYDYVLKYSLRLSITNGKVFRVSWIANSF 95
Cp1             ------------------------------------------------------------
Pa              PGALHDAIADVPRPALLGALSATALGFVILLGYEWSASRFAGVTLPMRSLATGGFSAFAI 120

Lm              TNIGGFGGVLGASLRASFYGKNAS-HKEILLAISKIALFLVSGLSIYCLVSLA-TLLIPG 161
Li              TNIGGFGGVLGASLRASFYGKNAS-HKEILLAISKIALFLVSGLSIYCLVSLT-TLLIPG 161
Cp2             ASLLGFGGATSLAFKQYFYGDYVDDKKKLLKEIGKIVALNLTGLSIVCCTYMG-IRISSW 158
Sa              NAIVGFGGFIGAGVRAMVYKNYTHDKKKLVHFISLILISMLTGLSLLSLLIVFHVFDASL 157
Sx              NAVVGFGGFIGASVRFLFYKNTTDDKKALFHTISIVLISMLTGLSLLSILVVIHVFDISH 157
Bc              NGFIGFGGLVGAGVRTMLYRPYIKENGKLIKSIAWMTTAFINGLAILSFLGLIGILDTSF 156
Ba              NGFIGFGGLVGAGVRTMLYRPYIKENGKLIKSIAWMTTAFINGLAILSFLGLIGILDTSF 156
Bs              NNVLGFGGLAGVGLRMMFYKEHTKDHKALVKGIAWLTSSMLLGLSVFSIFVAARVLPVDE 155
Cp1             ------------------------------------------------------------
Pa              GNAVGLSLLSGGSVRYRLYSRHGIGAAEIARMTLFASLSLGCALPVLAALAALCDLDDAA 180
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Lm              FADHFVNYWPWLLAGGLYFPILFTITKWKSKS---------------LFVDLPIKRELTL 206
Li              FADHFVNYWPWLLAGGLYFPILFTITKWKSKS---------------LFVDLPIKRELTL 206
Cp2             NNLGIIKYAIGIIA--LYAPGFILYSAYKYSK---------------TKDKLEFFSTLGI 201
Sa              ILDKITWVRWVLYVVSFFLPLFIIYSMVRPPD------------------KNNRFVGLYC 199
Sx              IFTPYPWVKWLMYVVALFLPIFVVFTIIKPVQ------------------KTHRLLGVYC 199
Bc              ILHEKPWLWPVLIFFALFVPIYIGFSKLKNRKP--------KQTEGQDEREEEKNPTVLY 208
Ba              ILHEKPWLWPVLIFFALFVPIYIGFSKLKNRKT--------KQAEGQDEGEEEKNPTVLY 208
Bs              VIHEKPWLWAVVIGFALILPLSLAVSKIKDRK-----------AGDEENADKVKNPIFAY 204
Cp1             ------------------------------------------------------------
Pa              SALHLPRALVAVIAIAVLSLAVGLVAFLARHRLPGERPSPDSLLVRLGRRSLRLPGLRLS 240

Lm              IIASLLEWGFAFGCFAIIGTLMGEPVDIFKVFPLFVIASVIGIASMVPGGVGTFDVVMIL 266
Li              IVASLLEWGFAFGCFAIIGVLMGEPVDIFKVFPLFVIASVIGIASMVPGGVGTFDVVMIL 266
Cp2             IFISFLEWLTTIILIYETLRITGASISVLNFLPIYIESAVVGMISMIPGGIGTFDLTFMT 261
Sa              TLVSCVEWLAAAVVLYFCGVIVDAHVSFMSFIAIFIIAALSGLVSFIPGGFGAFDLVVLL 259
Sx              TIVSGVEWFVAALVLYMSMAIVGVQIPFATFMGIFILAALSGLISFIPGGFGTFDLVVLL 259
Bc              SLVSLVEWVSAGIVMYVILILFGIDIEFQKFLGVYVIAALAGVVSLVPGGLGSFDLVFLT 268
Ba              SLVSLVEWVSAGIVMYVILILFGIDIEFQKFLGVYVIAALAGVVSLVPGGLGSFDLVFLT 268
Bs              IGASVVEWLMAGTVIYFALFAMGIHADIRYVFGVFVIAAIGGMISLVPGGFGSFDLLFLL 264
Cp1             ------------------------------------------------------------
Pa              LLQLLITALDVAAAATVLYLLLPETPPFAAFLLVYLLALAAGVLSHVPGGVGVFEAVLLA 300

Lm              GLSQLGVSQELALAWMLFYRIFYYIIPFVVGLLFFVQKAGKKVNDFLEG----------- 315
Li              GLSQLGVSQELALAWMLFYRIFYYIIPFIVGLLFFVQKAGKRLNDFLEG----------- 315
Cp2             GLEALGIPIEQTLLVIILYRISYYIVPALIGVLLFVHDFGGKINKKFNG----------- 310
Sa              GFKTLGVPEEKVLLMLLLYRFAYYFVPVIIALILSSFEFGTSAKKYIEGSKYFIPAKDVT 319
Sx              GLKALNVNEEAIVLGLSLYRFAYYLFPVLIALILSTFEFRSTAKRYWEDSRILVPVKDMT 319
Bc              GLGQYGIDTGVLLPAMLLYRLVYYILPFCLGLIFAAFEMTGVAIKKFEDKPFIAPALETT 328
Ba              GLGQYGIDTGVLLPAMLLYRLVYYILPFCLGLIFAAFEMTGVAIKKFEDKPFIAPALETT 328
Bs              GMEQLGYHQEAIVTSIVLYRLAYSFIPFILGLFFAAGDLTENTMKRLETNPRIAPAIETT 324
Cp1             --------------MWDSLKKSYRHLKNILGFVTDKRNYENIKKLLKN------------ 34
Pa              AFAG-QLGAAPLAAALLLYRLIYVVLPLLLACLLLLFLEARRLWVTRQA----------- 348
                                     :  *  .   :. .              :

Lm              -----LPLLFLQKVAHRFLVIFVYGSGLLLILSSAVPNAIYHVPFLYKIMPFNFLFTSQI 370
Li              -----LPLLFLQKVAHRFLVFFVYGSGLLLILSSAVPNAIYHVPFLYKIMPFNFLFTSQI 370
Cp2             -----LPYEIVSKVAYKIVVSLVFISGAIIVLSNIAPQYLLKIKLLKEILGKQVLGLSIG 365
Sa              SFLMSYQKDIIAKIPSLSLAILVFFTSMIFFVN-----NLTIVYDALYDGNHLTYYILLA 374
Sx              SLLGSYQKDIIARIPSFAIALLLLFTSLVFFLN-----NLTIIYDGLYDPNHYIYYIIVS 374
Bc              GVIWTLQRDFLGKLGSWASAALTVFAGLMVILSTILPTSINRAHALHILAPKHLIQFSFS 388
Ba              GVIWTLQRDFLGKLGSWASAALTVFAGLMVILSTILPTSINRAHALHILAPKHLIQFSFS 388
Bs              NVLLVVQRAVLVRILQGSLSLIVFVAGLIVLASVSLP--IDRLTVIPHIPRPALLLFN-G 381
Cp1             -------YKILSDISNIIVSVLVFLSGILLIISGIYPSIFYKIKFLDNIYSLSFLRFSHR 87
Pa              -------IRVASGFAAPILAILVFLSGVVLLFSGATPAIDTRLEHLGFLIPHRLIDASHL 401
                             .   .       :   :. :.. .

Lm              TIVAFGFLLLGLARGIECKTKKAYIITVIVLGCAIFNTLARVFSMKQAIFLGIVLLCLFL 430
Li              TIVAFGFLLLGLARGIECKTKKAYIITVIVLGCAIFNTLARVFSMKQAIFLGIVLLCLFL 430
Cp2             MSVVLGFLIMLAALMLKYRAKSIYKASMVLFILGIILSLTKGINPYELVFLIIVAYLLYL 425
Sa              IHTSACLLLLLNVVGIYKQSRRAIIFAMISILLITVATFFTYASYILITWLAIIFVLLIV 434
Sx              IHTCACLLLLLNVIGVYKLSKRAILFSIISVLFIFIATAYTYASFILLSWLTVIFILLLV 434
Bc              LSLTFGILLLILSRGIYYGTKRSYYMTIVSLIGAAIFNTLKGIDIEETFILLIVLAVLYM 448
Ba              LSLTFGILLLILSRGIYYGTKRSYYMTIISLIGAAVFNTLKGIDIEETFILLIVLAVLYM 448
Bs              LSLSSALILLILPIELYKRTKRSYTMAITALVGGFVFSFLKGLNISAIFVLPMIIVLLVL 441
Cp1             ASILIGLMLIMTSKEVFFKVKRAYYVTLTLLIVGGAFAFIKDLDYKEGIFILGVIILLIL 147
Pa              VASLIGVLCLLLAQGLRRRLSAAWALTLVLLLVGALLSLLKGFDWEEASLLSLTAALLAM 461
                      .: :     :          ::  .             .      :      * :

Lm              ARNEFYREK--LVYTWSKVIIDSIIFIVCLAGYI-VIGIYNSPNIKHSKEIPDYLRIASE 487
Li              ARNEFYREK--LVYTWSKVIIDSIIFIVCLAGYI-VIGIYNSPNIKHSKEIPDYLRIASE 487
Cp2             SKRMFYRDS--FVVSCKNTLIDSGILIASFSIYF-FILITFGTHLKYVGIVRKMPYKMAY 482
Sa              AFRRARRLK--RPVRMRNIVAMLLFSLFILYVN------HIFIAGTLYALDIYTIEMHTS 486
Sx              FYRRARVIK--RPFRYSKLLLSVITGAIILYIN------HLVIKSTFYSLEIYHIEMLTS 486
Bc              LRKRFVREK--MEVSLSDIVKVFIFLLITLYLYK-NLG-ILFAGAKEAFKPDFVVRNITQ 504
Ba              LRKRFVREK--MEVSLSDIVKVFIFLLITLYLYK-NLG-ILFAGAKEAFKPDFVVRNITQ 504
Bs              LKKQFVREQ--ASYTLGQLIFAVALFTVALFNYN-LIAGFIWDRMKKVLRHEYFVHSTSH 498
Cp1             SKKSFYRKS--IPIKVTKLSGILIVLSIVMIIFASFIHKFNIHFSKNYKYYIDFFHSTKG 205
Pa              FRRSFYRPSRLMEVPFSPLYVGASICVVGASVWLLLFANQDVHYSNQLWWQFALDADAPR 521
                  .     .                                       .
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Lm          HLWLVGFVGVFIAVVSLVIIYIYLSTTKEKLGSPFEAVKVREHLAKWG-GNEVSHTMFLR 546
Li          HLWLVGFVGVFIAVVSLVIIYIYLSTTKEKLGSPFEAVKVREHLAKWG-GNEVSHTMFLR 546
Cp2         KFGFIAFALVTVIYVAIYFLNIRRKIPVKTFDQCSEYIEK--IIEEYK-GDSLTHLVFLK 539
Sa          VLRYYFWLTILIIAIIIGMIAWLFDYQFSKVRISSKIEDCEEIINQYG-GNYLSHLIYSG 545
Sx          ILRYYFWITILLVAIIVGVIVWWFEYRYRSSNSRDNIATCESIIDKYN-GNYLSHLMYSG 545
Bc          VKRSALAAAFFVPTFLLIGSLIANRYRNEFPGQPANDKRLQNFLDEHG-GNVLSHLGFLG 563
Ba          VKRSALAAAFFVPTFLLIGSLIANRYRNEFPGQPANDKRLQNFLDEHG-GNVLSHLGFLG 563
Bs          ITHATIMAIIIVPLFFLIFTVVYHK-RTKPIGEKADPERLAAFLNEKG-GNALSHLGFLG 556
Cp1         YLRIALFTYISFIIFVIIWYLTMPKIEDDERYMDADLEKVSKFFKEIDYGTIFSHLVYLK 265
Pa          ALRAALGSCLLLLALALGWLLRAAPPAIREPN-AEELQRAARIIRHSD--QPDGGLALTG 578
                      . . . :                   .       : .

Lm          DKLLFWAAEGEVLFSYRIIADKMVIMGEPTGNMDKMEAAIEEVMMNADRFGYRPVFYEVR 606
Li          DKLLFWAANGEVLFSYRIIADKMVIMGEPTGNMEKMEDAIEEVMTNADRFGYRPVFYEVR 606
Cp2         DKYIYLNEDKDLFIQYEVYGDKLFVLGNPVGNNENLFREIEKFCEYADNYGYTPVFYQVN 599
Sa          DKQFFTNENKTAFLMYRYKASSLVVLGDPLGDENAFDELLEAFYNYAEYLGYDVIFYQVT 605
Sx          DKKFFINDNKDAFVMYRYHNNTYIILGDPIGNSESFYSLLEAFYKEAEYLGYDIIFYQVT 605
Bc          DKQFFFSSDGKALLLFSITGKRLVVLGDPIGDPSSYRTVLQEFLAEADRFGYICVFYQIE 623
Ba          DKQFFFSSDGKALLLFSITGKRLVVLGDPIGDPSSYRTVLQEFLAEADRFGYICVFYQIE 623
Bs          DKRFYFSSDGNALLLFGKIARRLVVLGDPSGQRESFPLVLEEFLNEAHQKGFSVLFYQIE 616
Cp1         DKKVFWANEGESLIMYSKYKDKIIVLGDPIATKENLYSCIEEFQAFTNLYGYDVVFYEIE 325
Pa          DKALLFHESDDAFLMYARRGRSMIALYDPIGPAMQRAELIWQFRDLCDLHHARPVFYQVR 638
            ** .    .   :. :       . : :* .        :  .    .      :**::
            D579 -> D579A, D579N

Lm          GTMIPYLHDHGFDFIKLGEEGFVDVQNFTMS--GKKKKGERALMNKLEREGYTFEIIEPP 664
Li          GTMIPYLHDHGFDFIKLGEEGFVDVQNFTMS--GKKKKGERALMNKLDREGYTFEIIQPP 664
Cp2         EEMISYLHSNGYDFMKIGEEAKVDVKEFKVV--GNKMKSLKTSRSKVTKEGYTFHMVEPP 657
Sa          DQHMPLYHNFGNQFFKLGEEAIIDLTQFSTS--GKKRRGFRATLNKFDELNISFEIIEPP 663
Sx          DKYMSLYHSFGNQFFKLGEEAVINLTSFTTS--GKKKRGLRATLNKLDDLGYSFEVLEPP 663
Bc          SKWMSLYHDFGYNFFKLGEEAVVDLNTFTIT--GKKRAGMRATFNRFEREGYTFSIHQPP 681
Ba          SKWMSLYHDFGYNFFKLGEEAVVDLNTFTIT--GKKRAGMRATFNRFEREGYTFSIHQPP 681
Bs          REDMALYHDFGYNFFKLGEEAYVDLNTFTLT--GKKKAGLRAINNRFEREEYTFHVDHPP 674
Cp1         EKNFSTYHDAGYYFFKLGEEARIDLEEFNLI--GSKKSAFRNTLRRVEREGYNFSIIEPP 383
Pa          AENLPFYMDIGLTALKLGEEARVDLLRFDLENKGKEMKDLRYTWNRGQRDGLALEFHEP- 697
               :.   . *   :*:***. :::  *     *.:    :    :       : . .*
                                  E657 -> E657D, E657Q
                                    E658 -> E658D, E658Q

Lm          FNHDTWTTLRAVSDEWLDGR--EEKGFSLGFFDTYYLEQAPIAIAKNGE-GTIVGFASMM 721
Li          FNNEMWKTLRAVSDEWLDGR--EEKGFSLGFFDTYYLEQADIALAKNAD-GTVVGFASMM 721
Cp2         FSREFLDYLKEISDEWLDGR--KEKGFSVGFFDEDYLNKAPIAILRDRE-GEIKAFANIM 714
Sa          FSTEFINELQHVSDLWLDNR--QEMHFSVGEFNEEYLSKAPIGVMRNEE-NEVIAFCSLM 720
Sx          FSQQMITDLKAISDDWLADK--NEMHFSVGSFDEHYISQAPIGVLKDNE-QSVIAFCTLM 720
Bc          FSDELFEELRKVSDAWLGGK--KEKSFSLGYFDREYISRAPIATLSDAD-GKIIAFTTFM 738
Ba          FSNELFEELRKVSDAWLGGK--KEKSFSLGYFDHEYISRAPIATLSDAD-GKIIAFTTFM 738
Bs          FSDAFLEELKQISDEWLGSK--KEKGFSLGFFDPSYLQKAPIAYMKNAE-GEIVAFANVM 731
Cp1         FNNEVVSQLKEISDKWLGDR--KEKGFSLGWFSEDYIQRSPIAILKNEEENKIMGFVTIM 441
Pa          -GQAPLDELKAISDAWLGGKQVREKGFSLGRFTPAYLNFFRIAIVRHQG--KPVAFANLL 754
             .      *: :** ** .:  .*  **:* *   *:.   *.   .       .* ..:
                                S709 -> S709A, S709N
                                 D710 -> D710A, D710N
                                          E720 -> E720Q
                                            S724 -> S724A, S724N

Lm          PSYTDE-MTSIDLMRYSKEAPSGIMDFLFINLFEKAKEDGFQTFNAGMAPLANVGESKYA 780
Li          PSYTDE-MTSIDLMRYSKEAPSGIMDFLFINLFEKAKEDGFQTFNAGMAPLANVGESKYA 780
Cp2         YMYDDE-SFSVDLMRFSKNTPRGVMDFMFINLIEYGKEKGYEIFNMGMAPLANVGLSKYA 773
Sa          PTYFND-AISVDLIRWLPELDLPLMDGLYLHMLLWSKEQGYTKFNMGMATLSNVGQLHYS 779
Sx          PTYYNG-VISVDLIRWKQDIELPLMDSLYLNMLLWSKDNNYEHFNMGMATLSNVGQIPYS 779
Bc          PVYQDG-SLSVDLMRYYPDAPSGIMDAIFIHLFQWAKENEYHSFNIGMAPLSNVGLSTQS 797
Ba          PVYQDG-SLSVDLMRYYPDAPSGIMDAIFIHLFQWAKENEYHSFNIGMAPLSNVGLSTQS 797
Bs          PMYQEG-EISVDLMRYRGDAPNGIMDALFIRMFLWAKEEGCTSFNMGMAPLANVGTAFTS 790
Cp1         DANDGGETVAIDLMRIDKDAPNASMDYLMLNLFLTFKEKGYKYFSLGEAPLSNVGFNTHS 501
Pa          ETDSRE-LASLDLMRVHPDAPKLTMEFLMLGLILHYKAQGHARFSLGMVPLAGLQPRRGA 813
                    ::**:*   :     *: : : ::   * .    *. * ..*:.:     :
´                   S763 -> S763A, S763N
```

```
Lm              FLGERLAGLVYRYSQGFYGFKGLRNFKSKYVTEWEQKFVAFRKRSSIAFTMLQLMILVGK 840
Li              FLGERLAGLVYRYSQGFYGFKGLRNFKSKYVTEWEQKFVAFRKRSSIAFTMLQLMILVGK 840
Cp2             FWNEKLALQFYENGQALYSFKGLRRFKEKFSHNWEYKYIAYRRNTSILITVIQAAIVCSR 833
Sa              YLRERLAGRVFEHFNGLYRFQGLRRYKSKYNPNWEPRFLVYRKDNSLWESLSKVMRVIRH 839
Sx              FYGERIAGRVFEHFNGLYRFQGLRRYKEKFNPKWEPRFLVYRKHQSLWVSMLKVMRVIRK 839
Bc              FWSERVAAAIFNNVRYTYSFSGLRHFKEKYKPAWSGKYLAFRKNHSLPITMLSVTKLIGK 857
Ba              FWSERVAAAIFNNVRYTYSFSGLRHFKEKYKPAWSGKYLAFRKNHSLPITMLSVTKLIGK 857
Bs              FWSERFAAVIFNNVRYMYSFSGLRAFKEKYKPEWRGKYLAYRKNRSLSVTMFLVTRLIGK 850
Cp1             HLQEKLARLVYNSGNIFYSFDGLRRYKSKFSPIWQPRYLAYPKFMSLPEVFINLCLLIAN 561
Pa              PLTQRLGALVFRRGEQFYNFQGLRRFKDKFQPDWEPRYLAVPAGLDPLVALADTAALIAG 873
                   ::..  .:.  .  * *.*** :*.*:   *  :::.     .    .     :

Lm              KRPLANSQVVLDFPLEEETKKPDSE 865
Li              KRPLANNQVVLDFPLNEEIEKPDSE 865
Cp2             NRNVDESIVVRNLKSLIK------- 851
Sa              K------------------------ 840
Sx              NN----------------------- 841
Bc              RKNS--------------------- 861
Ba              RKNS--------------------- 861
Bs              SKKDSV------------------- 856
Cp1             SKERVEKK----------------- 569
Pa              GLTGLVKR----------------- 881
```

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